Evolution in *Rana temporaria* Populations at a Small Geographical Range

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"Nothing in biology makes sense,

except in the light of evolution"

Theodosius Dobzhansky (1900-1975)

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SUMMARY

Evolution of natural populations is mainly shaped by genetic drift, gene flow, and natural selection. The final outcome, and whether populations will adapt to their living conditions, will depend on their relative strengths. These are in turn molded by population size and habitat.

In recent times, natural habitats have increasingly suffered from anthropogenic habitat destruction and fragmentation. As a consequence, terrestrial plant and animal populations are often becoming isolated and reduced in size. Some consequences of reduced population size are well understood, such as the enhanced impact of genetic drift and loss of genetic variation. Loss of genetic variation decreases the capacity of small populations to evolve and adapt to novel or changing environmental conditions. However, drift and selection may act synergistically in small populations, and the negative effects of small population size may be counteracted by gene flow, which provides new genetic variation. Effective gene flow depends on the organism's capacity to disperse, but also on the degree of population isolation (i.e. distances among patches and their connectivity). The impact of gene flow on adaptation is not yet clear, and can be seen as a constraining as well as a creative force.

The aim of this study was to understand how habitat and population size affect evolutionary forces, and thus to discover where and when selection, and ultimately adaptation, are favoured.

In order to understand the influence of population size on local adaptation, in **Chapter 1** I examined the adaptive response of a frog (*Rana temporaria*, Linnaeus) to habitat desiccation. Larvae originating from 16 populations of different sizes (small/large) and from different habitat types (temporary/permanent) were raised under two treatments (constant/decreasing water level) in a lab experiment. I

measured age and mass at metamorphosis, and survival during metamorphic climax. Larvae from large populations exhibited greater acceleration of larval period, and the habitat of the source populations was not important. Interestingly, populations from temporary and permanent wetlands performed differently after emergence. Generally, late metamorphs were heavily impacted by mortality, indicating strong selection favouring early metamorphosis. In particular, froglets from temporary ponds survived better if they were larger, whereas the contrary was true for individuals from permanent ponds. This may reflect an ability of larvae from temporary habitats to compensate for small body size despite a short developmental time, and also suggests that they are evolving a constitutive rather than a plastic response to desiccation, in order to better cope with regular drying. Moreover, considerable variation in larval period among populations within the constant water treatment suggests that other environmental components may also cause population divergence.

Comparison of divergence at neutral markers and quantitative traits provides an indirect tool to assess the strengths of natural selection and drift in shaping population differentiation. In **Chapter 2**, I assessed neutral and quantitative genetic differentiation of 16 populations that were chosen to represent two population sizes (small/large) and two habitats (sunny/shady). Neutral genetic differentiation, F_{ST}, was evaluated using eight microsatellite loci, whereas quantitative genetic differentiation, Q_{ST}, was inferred for life-history, morphological, and behavioural traits measured on larvae raised in a common-garden experiment. I found no evidence for phenotypic adaptation to canopy cover, as there was no systematic difference between habitats in quantitative traits. However, quantitative genetic differentiation of all traits was higher than neutral genetic differentiation, indicating that selection is acting in these populations. The difference between Q_{ST} and F_{ST} was non-random with respect to

canopy cover for two life-history traits and one morphological trait. Low levels of divergence were shown by populations originating from shady ponds, compared to populations from sunny ponds, which may indicate different selection pressures within the two habitats. Neither F_{ST}, Q_{ST}, nor the difference between them were related to population size. This implies a prominent role of gene flow, also confirmed by relatively low neutral differentiation (F_{ST} = 0.023) combined with significant isolation-by-distance. Thus, selection is probably not strong enough to overcome the effects of gene flow in this system.

The lack of a relationship between F_{ST} and population size in the second chapter indicated that the population counts used in that study did not accurately reflect genetic effective population size, N_e. Gene flow evidently plays a dominant role in this system. Thus, in **Chapter 3**, I assumed that genetic data from the previous 16 populations reflected their true long-term N_e, and asked whether N_e could be predicted on the basis of population counts. By combining landscape information with census size data, I assessed the contribution of surrounding populations to the genetic variation observed within the target populations and identified factors that affect gene flow. Genetic variation in the target population was unrelated to its population count, but was instead significantly correlated with the target count plus a weighted sum of the sizes of all surrounding populations within 2 km. Moreover, I found that dispersal decreased with distance; roads had a negative impact, and there was some evidence that forested land may promote dispersal. These results highlight the difficulty of identifying the limits of demes, even in cases such as frogs, in which populations appear to have discrete borders. The surrounding landscape has also to be considered in conservation and management.

In **conclusion**, I did not detect adaptation of frog populations to pond desiccation or canopy cover. However, I found that populations from temporary ponds may be evolving a constitutive rather than a plastic response to ephemeral habitats, and that selection within shady habitats is more uniform than within sunny habitats. From the first two experiments, there was indirect evidence that populations may respond to other environmental features than those under focus. The role of adaptive plasticity should not be forgotten, especially for generalist species such as *R. temporaria*, which breeds in nearly all types of water body. In fact, it has been shown that the evolution of plasticity will be favoured by both temporal and spatial heterogeneity. Moreover, gene flow seems to be relatively high, even though many populations appear to be quite isolated on a map. Dispersal in amphibians is thus crucial but still poorly understood. One consequence of high gene flow is that my measures of population sizes were unrelated to the extent of adaptation. It appears that true populations extend far beyond the borders of a specific wetland or pond, and immigration and emigration rates seem to be positively related to local population size. The question of how population size interacts with selection remains an important and unanswered question. While this will require a study system with less gene flow, these populations are suitable for answering other very interesting questions about the evolution of adaptive plasticity and the role of gene flow.

ZUSAMMENFASSUNG

Die Evolution natürlicher Populationen wird vor allem von den Faktoren Gendrift, Genfluss und natürliche Selektion voran getrieben. Das Resultat evolutionärer Prozesse und insbesondere die Fähigkeit von Populationen sich an die gegebenen Lebensbedingungen anzupassen, hängt von der relativen Stärke dieser einzelnen Faktoren ab. Diese wiederum werden massgeblich von der Populationsgrösse und vom Habitat beeinflusst.

Natürliche Lebensräume haben in den letzten Jahrzehnten zunehmend unter anthropogen verursachter Zerstörung und Fragmentierung gelitten. Infolgedessen werden Populationen terrestrischer Pflanzen und Tiere oft schrumpfen und in Isolation geraten. Die hieraus entstehenden Auswirkungen sind teilweise gut begriffen, wie etwa der relativ steigende Einfluss von Gendrift und die Abnahme der genetischer Vielfalt. Der Verlust genetischer Vielfalt mindert das evolutive Potenzial kleiner Populationen und somit deren Anpassungsfähigkeit an neue oder sich ändernde Lebensbedingungen. Gendrift und natürliche Selektion können auch synergistisch wirken und Genfluss kann durch neues genetisches Material den negativen Effekten, die mit kleinen Populationsgrössen einhergehen, entgegenwirken. Erfolgreicher Genfluss hängt von der Migrationsfähigkeit eines bestimmten Organismus ab, aber auch von dem Isolationsgrad der Populationen (d.h. den Distanzen zwischen deren Refugien und ihrer Konnektivität). Der Einfluss von Genfluss auf Anpassung ist noch nicht hinreichend klar, er kann vermutlich sowohl als kontraproduktiv als auch förderlich interpretiert werden.

Ziel dieser Studie war zu verstehen, in wie weit Habitateffekte und die Grösse von Populationen evolutionäre Prozesse beeinflussen, um dadurch Schlussfolgerungen ziehen zu können, unter welchen Umständen natürliche Selektion und somit

Adaptation begünstigt werden.

Um den Einfluss von Populationsgrössen auf deren Anpassungsfähigkeit zu erforschen, habe ich in **Kapitel 1** die adaptive Antwort vom Grasfrosch (*Rana temporaria*, Linnaeus) auf Habitataustrocknung untersucht. Kaulquappen, die von 16 unterschiedlichen, je entweder kleinen oder grossen, Populationen mit unterschiedlicher Habitatstruktur (temporäre versus permanente Gewässer) stammten, wurden jeweils unter den beiden Laborbedingungen konstanter, bzw. abnehmender Wasserpegel aufgezogen. Die Entwicklungszeit bis zur Metamorphose, das Gewicht zur Zeit der Metamorphose und die Überlebensrate während des Metamorphoseclimax (d.h. während der letzten Schritten der Metamorphose von der Kaulquappe zum Frosch) wurden dokumentiert. Froschlarven aus grossen Populationen wiesen eine kürzere Entwicklungszeit unter abnehmendem Wasserpegel auf, wobei die ursprüngliche Habitatstruktur keine Bedeutung hatte. Interessanterweise waren Unterschiede in der Überlebensrate bei Populationen von unterschiedlicher Herkunft festzustellen. Im Allgemeinen hatten Individuen, die später metamorphosierten geringere Überlebenschancen, was auf starke Selektion während früher Phasen der Metamorphose hinweist. Jungfrösche aus temporären Habitaten überlebten tendenziell besser wenn sie schwer waren, wohingegen Jungfrösche aus permanenten Habitaten eher überlebten, wenn sie leichter waren. Dieses Resultat könnte darauf hindeuten, dass Kaulquappen aus temporären Gewässern ihre Körpergrössen bei kürzerer Entwicklungszeit in gewisser Weise kompensieren können. Zudem legt dieser Befund nahe, dass Kaulquappen aus temporären Gewässern eine eher konstitutive statt plastische Form der evolutiven Reaktion auf wiederholte Austrocknungen entwickeln können. Allerdings zeugen die beträchtlichen Unterschiede in der Entwicklungszeit zwischen den

Populationen bei den Versuchsansätzen mit konstanten Wasserpegeln davon, dass auch andere Umweltfaktoren die Populationen diesbezüglich beeinflussen.

Der Vergleich der Differenzierung für neutrale genetische Marker und quantitative Merkmale liefert ein indirektes Mass, den Einfluss von natürlicher Selektion und Gendrift auf Populationen einzuschätzen. In **Kapitel 2** wurde die neutrale und quantitativ-genetische Differenzierung von 16 Grasfrosch-Populationen gemessen, welche nach den Kriterien Populationsgrösse (klein *versus* gross) und Habitatstruktur (sonnige *versus* schattige Gewässer) ausgewählt wurden. Die neutrale genetische Differenzierung, F_{ST}, wurde anhand von acht Mikrosatelliten-Loci ermittelt; die quantitativ-genetische Differenzierung, Q_{ST}, wurde für Lebenszyklus-Parameter, sowie morphologische und Verhaltens-Merkmale von in Mesokosmen unter gleichen experimentellen Bedingungen aufgezogenen Kaulquappen abgeschätzt. Es konnten keine Hinweise für phenotypische Anpassungen an den Beschattungsgrad gefunden werden, da es keine Zusammenhänge zwischen ursprünglichen Habitatstrukturen, aus denen die Kaulquappen stammten, und quantitativen Merkmalen während des experimentellen Ansatzes gab. Dennoch war die quantitativ-genetische Differenzierung aller Merkmale insgesamt höher als die Differenzierung über die neutralen genetischen Marker. Das bedeutet, dass natürliche Selektion auf diese Populationen einwirkt. In Bezug auf ursprüngliche Habitate konnte gezeigt werden, dass für zwei Lebenszyklus-Parameter und ein morphologisches Merkmal deutliche Zusammenhänge zwischen Q_{ST} und F_{ST} bestehen. Die Populationen aus schattigen Lebensräumen wiesen eine kleinere Divergenz im Vergleich zu Populationen aus sonnigen Teichen auf. Das könnte ein Zeichen dafür sein, dass innerhalb der unterschiedlichen Habitatstrukturen auch unterschiedliche Selektionsdrücke wirken. Weder F_{ST}, Q_{ST}, noch die Differenz zwischen diesen war mit dem Faktor

Populationsgrösse korreliert. Das bedeutet, dass Genfluss eine grosse Rolle in diesem System spielt, was auch von der relativ niedrigen Differenzierung der Mikrosatelliten-Daten (F_{ST} = 0.023) und von der signifikanten Korrelation zwischen der paarweisen genetischen Differenzierung und der Entfernung zwischen den Populationen bestätigt wird. Demzufolge ist natürliche Selektion vermutlich nicht stark genug, um den Effekten von Genfluss entgegenzuwirken.

Die fehlende Beziehung zwischen F_{ST} und Populationsgrösse im zweiten Kapitel wies darauf hin, dass die in jener Studie gebrauchten Populationszählungen nicht die effektive, genetische Populationsgrösse, N_e, präzis widerspiegeln. Offenbar spielt Genfluss eine dominierende Rolle in diesem System. Unter der Annahme, dass die genetischen Daten den effektiven Populationsgrössen N_e der 16 untersuchten Populationen entsprechen, stellte sich die Frage, ob N_e anhand von Populationszählungen vorhergesagt werden kann. Die Ermittlung des Einflusses auf die genetische Variation der Ziel-Populationen durch deren benachbarte Populationen wurde durch die Berücksichtigung von Landschaftselementen und Zählungsdaten ergänzt, um Faktoren, die Genfluss beeinflussen, zu identifizieren. Die genetische Variation innerhalb Zielpopulationen war nicht ausschliesslich mit deren Grösse gemessen an den Zählungen der Gelege korreliert, sondern hing auch wesentlich von den Grössen der umliegenden Populationen innerhalb eines 2 Kilometer Radius ab. Die Migration nahm auch mit steigender Entfernung ab; Strassen hatten einen negativen Einfluss auf die Stärke des Genflusses, während anderseits bewaldete Landschaften diesen fördern zu scheinen. Diese Resultate zeigen die Schwierigkeit auf, die dabei besteht, die Grenzen der Ausbreitungsgebiete von Populationen festzulegen. Selbst wenn diese wie im Fall von Fröschen klar abgegrenzt zu sein scheinen, sollte gerade im Zuge von Natur- und

Artenschutzbestrebungen auch grundsätzlich die an einen augenscheinlichen Lebensraum einer bestimmten Population grenzende Landschaft berücksichtigt werden.

Als **Fazit** lässt sich zusammenfassen, dass keine Anpassungen an Austrocknung oder Gewässerbeschattung aufgedeckt werden konnten. Allerdings fand ich heraus, dass Populationen aus temporären Gewässern mit einer konstitutiven anstatt einer plastischen evolutiven Antwort auf Habitataustrocknung reagieren könnten und weiterhin, dass Selektion innerhalb schattiger Feuchtgebiete uniformer ist als die in sonnigen Habitaten. Aus den ersten zwei Experimenten ergaben sich indirekte Hinweise darauf, dass auch andere, nicht untersuchte Umweltfaktoren einen Einfluss auf natürliche Populationen haben können. Die Rolle von adaptiver Plastizität sollte nicht vernachlässigt werden, vor allem für Generalisten wie den Grasfrosch, der nicht sehr wählerisch bei der Wahl seiner Brutgewässer ist. In der Tat konnten andere Forscher zeigen, dass die Evolution von Plastizität durch zeitliche und räumliche Heterogenität voran getrieben wird. Ausserdem scheint Genfluss in diesem Forschungssystem von grosser Bedeutung zu sein, auch wenn viele Populationen auf einer Karte eher isoliert erscheinen mögen. Amphibien-Migration ist somit äusserst wichtig aber leider bis heute sehr wenig verstanden. Eine Folge von starkem Genfluss war, dass meine Messungen der Populationsgrössen nicht mit dem Ausmass an Anpassung korreliert waren. Es scheint, dass natürliche Grasfrosch-Populationen sich auch weit ausserhalb der Grenzen eines bestimmten Feuchtgebietes oder Teiches aufhalten und verbreiten können und somit Immigrations- und Emigrationsraten mit der Populationsgrösse positiv korreliert sind. In welcher Relation Populationsgrösse und natürliche Selektion zueinander stehen bleibt eine wichtige, aber unbeantwortete Frage. Während diese Fragestellung ein

System mit geringerem Genfluss benötigen würde, eignen sich Grasfrösche jedoch für andere spannende Fragen in der Biologie, zum Beispiel zur Erörterung der Evolution von adaptiver Plastizität und der Rolle von Genfluss.

GENERAL INTRODUCTION

Adaptive evolution in natural populations

The term evolution refers to any change in the heritable characteristics of natural populations from generation to generation. The theory of adaptive evolution was formulated by Charles Darwin, and became well-known with the publication of his book “The origin of species” in 1859 (Darwin, 1859). Based on detailed observations of plants and animals, Darwin postulated that natural populations evolve across generations through natural selection. The principles of evolutionary theory were later complemented by Fisher (1930), Wright (1931), Haldane (1932), and others, who developed the basics of population and quantitative genetics, and discovered their roles in evolutionary processes.

The major drivers of evolution are mutation, genetic drift, natural selection, and gene flow. Mutations, i.e. heritable changes in DNA sequences, and genetic drift, i.e. random fluctuations in allele frequencies, are processes that occur by chance. Gene flow happens as a result of dispersing individuals, which succeed in reproducing in their new population, and in this way contribute to its gene pool. Thus, these three forces act on the genetic level. The underlying genetic material of an individual corresponds to its genotype, which is expressed as its phenotype, i.e. its observable characteristics. Natural selection acts directly on phenotypes, but ultimately on genotypes (through phenotypes) and changes allele frequencies within and among populations. In theory, natural selection relies on the following three assumptions: the variation in phenotypes is heritable, there is variation in reproductive success (also termed fitness), and both factors are correlated. The relative strengths of these forces will determine the course of evolution, and whether adaptation of a particular population to its local habitat will occur.

A special case of adaptation is adaptive phenotypic plasticity, which is defined as a given genotype being able to produce different phenotypes depending on the surrounding environment. Adaptive plasticity can evolve in environments that are heterogeneous on either spatial or temporal scales.

Effects of habitat and population size

In recent decades, natural habitats have increasingly suffered from man-made disturbance. In fact, the intensification of agricultural practices to feed the constantly growing human population has led to widespread habitat loss, degradation and fragmentation (Vitousek *et al.*, 1997). As a consequence, natural populations of terrestrial plants and animals are often reduced in size and become isolated (Saunders *et al.*, 1991; Reed, 2004).

Reduction in population size is associated with enhanced importance of genetic drift (Kimura, 1955) and reduced genetic diversity (Frankham, 1996). Drift causes a loss of heterozygosity at a rate of $1/[2*N_e]$ per generation, where N_e refers to effective population size (Kimura, 1955). Therefore, genetic drift is especially important in small populations. Not only does drift result in random fixation of alleles at the rate given above, but it can also affect population fitness (Reed and Frankham, 2003). Small and isolated populations have an enhanced probability of extinction, because they are more susceptible to environmental and demographic stochasticity (Spielman *et al.*, 2004). Thus, reduction in population size may finally decrease the adaptive potential of the population (Willi *et al.*, 2006).

On the other hand, the negative impacts of reduced population size can be counteracted by gene flow, thus providing sink populations with new genetic variation. The amount of gene flow depends on the dispersal ability of the individuals, but also on the degree of isolation of the populations, which incorporates both the

distances among patches and the habitat that connects them. Thus, it depends on local habitat and larger landscape features: continuous landscape or corridors can favour gene flow, whereas barriers can hinder it. With respect to adaptation, the effects of gene flow are not yet clear. Gene flow can be viewed as a constraining force by preventing local adaptation, but also as a creative force by spreading new genes (Slatkin, 1987; Garant *et al.*, 2007). Moreover, if gene flow occurs among populations inhabiting similar habitats, the potential for adaptation to features of that habitat will not be hampered. Additionally, it has been shown that gene flow can also promote the evolution of adaptive plasticity (Lind and Johansson, 2007; Lind *et al.*, 2011).

The outcome of all evolutionary forces – i.e. mutation, genetic drift, selection, and gene flow and thus population adaptation – will finally depend on their relative importance and strength. These are ultimately dependent on population size and habitat. A better knowledge of how these two factors influence evolutionary forces, and thus detecting where and when selection and adaptation are favoured, will have major implications for understanding the evolutionary potential of endangered species and improving conservation management.

Study system

Worldwide, 32% of amphibian species are on the Red List, which by far exceeds the proportion of mammals and birds (Baillie *et al.*, 2004). Amphibians are threatened by various factors, including UV radiation, pollutants, and emerging infectious diseases (Collins and Storfer, 2003), but they are especially affected by habitat fragmentation (Cushman, 2006). Habitat fragmentation involves not only landscape changes, but also physical changes in microclimate (Saunders *et al.*, 1991). Disturbances in both landscape and microclimate particularly affect amphibians, because most of them are

exposed to terrestrial and aquatic habitats during different stages of their life cycles, and because they have highly permeable skin, which makes them very sensitive to environmental changes (Alford and Richards, 1999).

My research concerns the common frog, *Rana temporaria* (Linnaeus), a widespread European amphibian that is ubiquitous in my study area (750 km^2 between Zurich and Andelfingen, in northern Switzerland). This species breeds in a variety of different habitats, encompassing diverse water bodies (ranging from small puddles or ditches over middle-sized ponds to larger lakes), which differ in hydroperiod (temporary vs. permanent) and in surrounding habitat structure (open- vs. close-canopy cover). Thus, *R. temporaria* can be considered a good potential migrant compared to other more specialized species. Although *R. temporaria* is currently not endangered, natural populations exhibit very heterogeneous population sizes, including small populations of only a few dozen females and large populations of several thousands females within a single breeding site (as inferred from clutch counts). These population features make it a perfect species and system to address my questions, as detailed below.

Thesis outline

This study aimed at understanding the forces of drift, selection, and gene flow, and ultimately adaptation, and how these are influenced by population size and habitat. Using two different approaches I assessed the extent of adaptation related to population size. By studying genetic variation at neutral markers and in quantitative traits within and among populations, I explored the association between neutral and quantitative trait divergence and population size, and how this association relates to habitat. Finally, by linking genetic variation, demographic data and landscape features, I quantified gene flow and identified barriers to gene flow.

In order to understand how population size influences local adaptation, in **Chapter 1** I investigated adaptive response to habitat desiccation in larvae originating from 16 populations of different sizes (small/large) and of different pond origin (temporary/permanent). In a lab experiment, I tested the effects of two treatments (constant/decreasing water level) on developmental time and size at metamorphosis. I expected the greatest acceleration in development under desiccation in larvae from large populations and from temporary ponds, because adaptive plasticity is expected to evolve in heterogeneous environments (Bradshaw and Hardwick, 1989; Moran, 1992), and temporary ponds are more variable in hydroperiod than permanent ponds (Newman, 1989; Brooks and Hayashi, 2002).

In **Chapter 2**, I assessed neutral and quantitative genetic differentiation of 16 populations in relation to population size (small/large) and habitat (sunny/shady). Comparison of divergence at neutral markers and quantitative traits provides an indirect tool to assess the strengths of natural selection and drift in shaping population differentiation (Wright, 1951; Rogers and Harpending, 1983). The neutral genetic differentiation index, F_{ST} (Wright, 1951; Nei, 1987), reflects the action of drift and gene flow. An alternative and analogous index measuring population differentiation at quantitative phenotypic traits, Q_{ST} (Rogers and Harpending, 1983; Spitze, 1993), reflects the action of drift, gene flow and selection. Thus, comparing Q_{ST} and F_{ST} provides indirect information about the power and direction of selection promoting differences among populations, as well as on which quantitative traits selection is acting. Neutral genetic variation was evaluated using eight variable microsatellite loci, whereas quantitative genetic differentiation was inferred for life-history, morphological, and behavioural traits measured on larvae raised in a common-garden experiment. I expected that, irrespective of the habitat of origin, small populations would be more divergent from one another than large populations

at neutral markers and quantitative traits due to higher drift. Also, I hypothesized enhanced population divergence due to habitat, with selection acting more strongly on populations in shady ponds than on those in sunny ponds, because the former may represent harsher conditions for larvae. This is expected to result in lower among-population differentiation for closed-canopy populations than for open-canopy ones. This effect could be more important for large populations, where selection should be more efficient, according to theory.

A positive relationship between effective population size N_e and genetic variation is predicted by neutral theory (Kimura, 1983) and confirmed by empirical studies (reviews in Soulé, 1976; Frankham, 1996). Therefore, in **Chapter 3**, I used genetic data from several frog populations (under the assumption that these accurately reflect the true N_e) and estimated the contribution of surrounding populations to the maintenance of genetic variation in the target populations. I combined genetic data, demographic data and important landscape features (i.e. barriers and land uses) that occur among target ponds and other breeding sites within a 2-km radius. A model-comparison approach identified the factors that promote or hinder gene flow among frog populations.

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ADAPTIVE RESPONSE OF AMPHIBIANS TO HYDROPERIOD: WHEN DOES POPULATION SIZE MATTER?

Abstract

I investigated the adaptive response to habitat desiccation in larvae of the common frog (*Rana temporaria*, Linnaeus) to compare the level of adaptation between small and large populations originating from temporary and permanent water bodies. In a lab experiment I tested the effect of two treatments, constant and decreasing water level, on age and size at metamorphosis, and survival during metamorphic climax. I found greater acceleration of development in larvae from large populations than in those from small populations, independently of the hydroperiod of the source populations. On the other hand, all tadpoles became smaller when exposed to decreasing water levels, indicating that the mass loss may have been caused by an indirect effect of the treatment, rather than an unavoidable trade-off between development and body size. Interestingly, populations from different hydroperiod regimes performed differently after emergence. In general, post-metamorphic mortality especially impacted late individuals, suggesting strong selection favouring early metamorphosis. Specifically, froglets from temporary ponds survived better if they had higher mass, whereas the opposite was true for metamorphs from permanent ponds. This may imply an ability of temporary-pond tadpoles to compensate for small body size despite a short developmental time, and also suggests that they are evolving a constitutive rather than a plastic response to desiccation. Considerable heterogeneity among populations in larval period length within the constant water treatment suggests that environmental components other than hydroperiod may also probably cause population divergence.

Introduction

Habitat loss and fragmentation can have major impacts on natural populations, causing isolation and reduction in population size (Vitousek *et al.*, 1997; Reed, 2004; Cushman, 2006). Some consequences of fragmentation are well understood, such as the enhanced importance of genetic drift and reduced genetic diversity (Frankham, 1996; Reed and Frankham, 2003; Spielman *et al.*, 2004). But the implications of small population size and isolation for the adaptive potential of populations are less certain. In the 1930s, Fisher (1930) argued that adaptation takes place primarily in large populations with high genetic variation and low genetic drift, refining existing adaptations by natural selection. At the same time, Wright (1931) believed that adaptation could be facilitated within small populations through the combined action of drift and selection. Although Fisher was correct in theory that selection is more effective in large populations, parts of Wright's ideas have also been confirmed. For example, drift and selection may act synergistically in small populations (e.g. Wade and Goodnight, 1998; Willi *et al.*, 2007). However, field observations and artificial selection experiments have generally failed to support Wright's overall model, known as the shifting balance theory (Coyne *et al.*, 1997). This leaves me with the expectation that adaptation will be more pronounced in large populations.

Studies of local adaptation that account for population size have reported conflicting results. As reviewed by Leimu and Fischer (2008), local adaptation in plants is not ubiquitous, and is more probable in large than in small populations. Nevertheless, some other studies have found evidence for local adaptation despite small population size. For example, small populations of grayling (*Thymallus thymallus*, Salmonidae) that have existed for only about 100 years show very clearly diversifying selection producing adaptation to the local water temperature (Koskinen

et al., 2002). Also the rare sapphire rockcress (*Arabis fecunda*, Brassicaceae), occurring in very small populations, expresses differences in traits involved in drought tolerance that accord with local moisture levels (McKay *et al.*, 2001).

Population size has not yet been incorporated into studies of amphibian metamorphosis, although there are many cases of adaptive variation in metamorphic timing in response to environmental factors. For example, species occurring in permanent water bodies have long larval periods, and some amphibians can respond to pond desiccation by accelerating metamorphosis (Newman, 1992; Denver *et al.*, 1998; but see Brady and Griffiths, 2000). Patterns of population differentiation in developmental plasticity indicate that metamorphic timing can be locally adapted to desiccation risk (e.g. Laurila *et al.*, 2002; Merilä *et al.*, 2004), but it is not known whether and to what extent population size may influence adaptation.

Accelerating development in an ephemeral habitat may involve a trade-off with size at metamorphosis, because early emergence usually entails reduced body size (Travis, 1984). On the one hand, shorter developmental time provides an opportunity to escape from habitats that are temporary, thus improving early survival. On the other hand, rapid development leads to smaller size at metamorphosis, which in turn decreases juvenile survival and adult reproduction (Berven and Gill, 1983). This trade-off suggests that amphibian populations exposed to habitats of different duration will show differences in larval period and body size at emergence, and possibly in plasticity in these traits. Populations occupying temporary wetlands should develop more rapidly and accept a smaller size at metamorphosis; they may also exhibit greater plasticity induced by habitat degradation, assuming that plasticity is costly.

In order to understand how population size influences local adaptation, I investigated adaptive response to habitat desiccation in larvae of the common frog

(*Rana temporaria*, Linnaeus), originating from temporary and permanent wetland populations of different sizes.

In a lab experiment I tested the effect of two treatments, constant and decreasing water level, on developmental time and size at metamorphosis. I expected large populations from temporary ponds to show the greatest acceleration in development under desiccation because adaptive plasticity is expected to evolve in heterogeneous environments (Bradshaw and Hardwick, 1989; Moran, 1992), and temporary ponds are more variable in hydroperiod than permanent ponds (Newman, 1989; Brooks and Hayashi, 2002). At the same time, shorter developmental time may be coupled with smaller size at metamorphosis.

Material & Methods

Study species and populations

Rana temporaria (Linnaeus) is the most widespread amphibian in Europe, with a very broad habitat range: it can be found from sea level to > 2000m in the Alps, from Italy to above the Arctic Circle, and from small puddles to large lakes (Nöllert and Nöllert, 1992). Since it occurs in such variable habitats, *R. temporaria* shows broad developmental plasticity, including plasticity induced by habitat drying. When exposed to lowering water level, tadpoles accelerate development (e.g. Laurila *et al.*, 2002; Merilä *et al.*, 2004).

In March 2007, I collected five freshly laid *R. temporaria* egg clutches from each of 16 populations differing in population size (small and large) and hydroperiod (temporary and permanent). Clutches were chosen haphazardly, but came from different regions of the pond or different parts of the spawning aggregation to reduce the likelihood that any single male sired more than one clutch. Ponds were situated in northern Switzerland, in a total area of approximately 750 km^2 (Fig. 1; exact locations

are given in Appendix A). The eggs were held outside in small tanks until several days after hatching.

Population size was estimated from clutch counts during March 1997-2009. Small populations ranged from 20 to 160 egg clutches (mean = 72), whereas large populations from 670 to 3'183 (mean = 1'248). Pond permanence was calculated with a Principal Component Analysis on the correlation matrix of maximum surface area and depth of the pond, and the proportion of years during which it dried between 1997 and 2003. Temporary ponds dried out during at least some years and scored low on the first principal component (mean = -0.6, min = -2.9, max = 0.3). Permanent ponds always had water and had a positive score (mean = 1.3, min = 0.4, max = 1.9). I included a few ponds for which population size and drying records were not available at that time, but in these cases the population size and hydroperiod were confirmed from observations between 2007 and 2009.

Experimental design

The experiment had a 2 × 2 × 2 factorial block design. The three factors were population size (small/large), pond type (temporary/permanent), and water level in the lab (constant/drying). There were four source populations for every combination of population size and pond type, five families from each population, and three replicate tadpoles in each family/treatment combination. The experiment began on 4 April 2007, when tadpoles had just reached stage 25 (after Gosner, 1960), i.e. when the external gills atrophied completely (McDiarmid and Altig, 1999, pp. 10, 105).

The laboratory room was maintained at 21-22°C, with a 12:12 light:dark cycle shifted to 14:10 after two weeks to mimic natural day length. Experimental units were opaque plastic tubs (20 x 11.5 x 7.5 cm) with 0.8 liter water, each containing a single tadpole. I also added one beech leaf (*Fagus sylvatica*) in order to provide some shelter. Blocks were laboratory shelves. I fed tadpoles *ad libitum* with ground rabbit

food. Water was changed every fourth day, and kept constant for the first two weeks. Thereafter, water level in the decreasing treatment was reduced by 30% at every water change (i.e. every 4 days), until it reached 50 ml (corresponding to a depth of 0.2 cm) after 48 days, at which point most tadpoles had reached metamorphosis and the experiment was stopped.

For each individual, I recorded mass at the beginning of the experiment, age and size at early metamorphosis (forelimb emergence; stage 42), and age and size a few days later at complete tail resorption (stage 46, when metamorphic climax ends and the animal becomes a juvenile frog). I checked for metamorphs twice a day, and age was calculated as the number of days from the start of the experiment.

Statistical analysis

I began by testing for variation in initial tadpole mass, because egg size, and therefore hatchling size, may correlate with developmental rate and mass at metamorphosis (Kaplan, 1998). Hatchling mass differed among populations (log-likelihood test between models, *L-ratio* = 35.399, $P < 0.001$), but not between treatments within populations (Welch two sample t-test: all 16 comparisons n.s.). There was no association between hatchling mass and population size ($F_{1,12} = 0.08$, $P = 0.7782$) or pond type ($F_{1,12} = 2.65$, $P = 0.1293$), and the interaction between these two factors was not significant ($F_{1,12} = 0.43$, $P = 0.5231$).

The response variables were age and mass at stage 42, and survival between stage 42 and 46; age was square-root transformed before analysis to improve normality. The data were analyzed using linear mixed-effects models in which the fixed factors were water level treatment, block (laboratory shelves), and the size and type of the source populations. Population and family (nested within population) were treated as random factors. First, I analyzed age and mass at metamorphosis in a mixed-model ANOVA that included initial mass as a covariate to determine whether it

influenced metamorphic traits. I focused on stage 42 because nearly half of all individuals died between the beginning and end of metamorphic climax (between stages 42 and 46). I also inspected genetic correlations based on family means to evaluate how initial mass, age and mass at metamorphosis, and plasticity in metamorphic performance, relate to each other. These correlations may reflect constraints on the independent evolution of these life-history traits.

Survival between stages 42 and 46 was analyzed in a generalized linear mixed-effects model with a binary response. The objective was to investigate factors responsible for high mortality and to estimate selection acting on metamorphic traits. I used a multi-model inference approach (Grueber *et al.*, 2011; Symonds and Moussalli, 2011). A global model started with all fixed effects: pond type, population size, treatment, standardized age and mass at stage 42, and all two-way interactions among them. Standardization was done following Gelman's approach (2008). The random part of the model included the intercepts of population and genotype, and the slopes the fixed effects within population and genotype. This model provided an adequate fit to the data, reflected in the high correlation between fitted and observed values (R^2 = 0.36, df = 441, $p < 0.0001$) and modest over-dispersion ($\hat{c}$ = 1.274). I then generated a complete submodel set, retained those models within 2 AIC units of the best model, and estimated parameter values by model averaging ("natural averaging" method, where the parameters are averaged only over models in which they appear).

Statistical tests were conducted in R 2.13.0 (R Development Core Team, 2010), with the packages "nlme" (for the mixed-model ANOVA, Pinheiro *et al.*, 2009), "lme4" (for the mixed-model with binary data, Bates *et al.*, 2011), "arm" (for standardizing, Gelman *et al.*, 2011) and "MuMIn" (for multi-model inference, Barton, 2011).

Results

Age and mass at metamorphosis

Water level treatment had significant effects on both age and mass at metamorphosis (Table 1), reflecting faster development and reduced mass under decreasing water level (Fig. 2). A significant interaction between treatment and population size for age at metamorphosis occurred because tadpoles from large populations showed a much greater reduction in development time under desiccation than did tadpoles from small populations (Fig. 2a). The same interaction was not present for mass at metamorphosis, as all population kinds showed nearly the same reduction in body size under decreasing water level (Fig. 2b). Within the constant water treatment, tadpoles from small populations had the same mass at metamorphosis as those from large populations, despite having a slightly shorter developmental time. Initial mass had a strong negative effect on age at metamorphosis, but no effect on mass (Table 1). Excluding initial mass from the model did not change the main results (data not shown). There was appreciable genetic variation in age and mass at metamorphosis both within and among populations, as indicated by the importance of the random effects of family and population (Table 1).

Although I hypothesized that populations from temporary ponds would show a greater plastic response to decreasing water, there was in fact no interaction between treatment and pond type at stage 42. The expected pattern was present only at stage 46, when tadpoles from temporary ponds showed the greatest reduction in larval period and the largest decrease in mass under drying conditions (Fig. 2c and 2d). This pattern was not significant because high mortality between stages 42 and 46 weakened statistical power.

Tadpoles with longer development time under constant water level were those more able to shorten their larval period in response to treatment, as evidenced by a positive genetic correlation between the mean degree of plasticity (percentage reduction in developmental time between treatments) and the mean age at metamorphosis in the constant treatment (r = 0.231, n = 80, p = 0.039). On the other hand, developmental time was negatively associated with mass at metamorphosis, especially in the decreasing water treatment (constant water: r = -0.211, n = 80, p = 0.060; decreasing water: r = -0.702, n = 80, p < 0.001).

Survival during tail resorption

Metamorphs from temporary ponds had about 40% survival between stages 42 and 46, whereas those from permanent ponds roughly 60% (Fig. 3).

There was no overall best model to explain survival during tail resorption; five models were within 2 AIC units of the best model (Table 2). Parameter estimates suggested that survival was significantly influenced by age at stage 42 and by the treatment-by-type and mass-by-type interactions. Earlier metamorphs survived better (Fig. 3a), and those from permanent ponds survived especially well in the decreasing water treatment (Fig. 3), as also suggested by the significant treatment-by-type interaction (Table 2). Mass at stage 42 had opposite effects for temporary and permanent population types: in temporary pond populations higher mass improved survival, whereas in permanent pond populations lighter metamorphs had greater survival (Fig. 3b). Although the population size term appeared in two of the top six models, and froglets from small populations survived about 5% worse than those from large populations (data not shown), this effect was not significant.

Discussion

This study explored the importance of population size for the degree of adaptation to hydroperiod in *Rana temporaria* populations. There were important differences among populations in the ability to respond to desiccation. Animals from large populations showed a greater acceleration of metamorphosis than those from small populations, but the hydroperiod of the source pond was not important. This contradicts previous studies that found more plasticity in populations that often experience pond drying than in populations that do not (Laurila *et al.*, 2002; Merilä *et al.*, 2004). But, as in the earlier studies, I found that an overall time constraint may explain the lack of response by individuals from small populations. In general, populations with the longest development time under constant conditions reacted most strongly to desiccation. It appears that *R. temporaria* was unable to reduce the larval period to less than about 33 days under the conditions of my experiment.

The outcome was different when evaluated at the late metamorphic stage of tail resorption, because survival between forelimb emergence and tail resorption was non-random with respect to population type, water level treatment, and metamorphic phenotype (i.e., age and mass). When evaluated at the froglet stage, populations from temporary ponds accelerated development most strongly. Temporary-pond tadpoles that emerged late survived poorly over the following days, and this left only the earliest metamorphs, which were also heavier, among those reaching stage 46. For permanent-pond tadpoles, this same trend was present but much less strong, so that the survivors to stage 46 included a mixture of early and late metamorphs. Many studies of amphibian larval performance evaluate survival and condition when the forelimbs first appear (Walsh, 2010), but my results suggest that events occurring shortly thereafter can shift the outcome importantly. In the end, neither stage 42 nor

stage 46 represents a valid end-point for assessing individual fitness, and it may be worthwhile to include both.

Development rate in amphibian larvae may evolve in response to habitat features other than hydroperiod, including both biotic and abiotic factors, which can interact in complex ways (Denver, 1997). Indeed, considerable heterogeneity among populations in larval period length within the constant water treatment suggests that other unknown causes of population divergence exist. I also observed a constraint on developmental plasticity, identified by a positive genetic correlation between the degree of plasticity in development time and age at metamorphosis under constant water level. This suggests that populations with relatively short developmental time are constrained from accelerating it further. This is probably due to physiological limitations on differentiation rate, and not because acceleration would entail an unacceptable reduction in body size (Wilbur and Collins, 1973), as I found a negative relationship between age and mass at metamorphosis. In my study, this had clear implications for small populations, in which tadpoles had short larval periods under constant water and were unable to accelerate development in the drying treatment. A possible explanation for this is that constitutively high development rate has evolved in these populations in response to cold temperatures. Indeed, the four permanent ponds from which small populations originated are also relatively deep and forested, and therefore probably colder than the permanent ponds inhabited by large populations. The early metamorphosis visible in Fig. 2a may simply reflect countergradient variation (Berven *et al.*, 1979; Conover and Schultz, 1995). Similar results come from studies comparing *R. temporaria* populations along a latitudinal gradient, where northern populations exhibited more rapid development (Laugen *et al.*, 2003) and also no ability to respond to a decreasing water treatment (Laurila *et al.*, 2002; Merilä *et al.*, 2004). However, the association in my experiment between

development rate and temperature in the source pond is speculative because I have no data on water temperatures and a re-analysis of the data reveals no effect of canopy cover when substituted for population size.

A second habitat feature not included in my design is suggested by models of the evolution of phenotypic plasticity, which highlight the importance of temporal and spatial heterogeneity in the environment (Stearns, 1989; Schlichting and Pigliucci, 1998). In this context, year-to-year variation in drying may be more important than average hydroperiod in selecting for the ability to accelerate development. Some of my source ponds dry essentially every year, and are thus no more variable in hydroperiod than large permanent lakes. Of course, this issue affects my predictions for plasticity – and not average values – of development rate.

My tadpoles experienced a high post-emergent death rate, which is fairly common – but rarely reported – in tadpoles reared under laboratory conditions (Alford and Harris, 1988, Ficetola G.F., Baumgartner S., Egea Serrano A., Laurila A., personal communications). I speculate that many other studies on response to hydroperiod have experienced this problem because they typically report age and mass only at stage 42, even though mass at stage 46 is more stable and repeatable (Travis, 1980). My results show that, even if mortality is due to unsuitable rearing conditions (e.g. light, temperature, food), it can occur non-randomly with respect to treatment and substantially affect the results. It may also provide interesting information that could otherwise have been lost. Populations from different hydroperiod regimes performed differently after emergence, with temporary pond froglets surviving poorly compared to populations from permanent ponds. More generally, post-metamorphic mortality especially impacted late individuals, creating strong selection favouring early metamorphosis. This was especially true for individuals from temporary ponds, which faced nearly no chance of survival if they emerged late. Moreover,

metamorphs from temporary ponds survived better if they had higher mass, whereas the opposite was true for individuals from permanent pond populations. This may reflect an ability of temporary-pond tadpoles to compensate for small body size despite a short developmental time. Semlitsch *et al*. (1988) reported similar results in a salamander (*Ambystoma talpoideum*): when the pond dried late, there was no difference in mean body size between early and late individuals, which suggests higher fitness for the fast-growing early metamorphs. Perhaps this pattern arose from a history of more effective natural selection on post-metamorphic performance in early metamorphs from temporary ponds; late individuals are killed by pond-drying during some years and are therefore less visible to selection after stage 42. The short larval period and large size of temporary-pond individuals also suggests that they are evolving a constitutive rather than plastic response to drying, in order to better cope with regular desiccation.

Larval period at constant water level was negatively associated with mass at metamorphosis. This suggests that selection for shorter developmental time will also lead to selection for larger body size. Of course, froglets would benefit from emerging earlier and larger (Berven and Gill, 1983). On the other hand, tadpoles in all populations became smaller when exposed to decreasing water levels, including even those populations that did not show accelerated development. This may have been caused by an indirect effect of the treatment, rather than an unavoidable trade-off between development and body size. In fact, it has been shown that tadpoles react to reduced water volume by feeding less (Denver *et al.*, 1998). Hence, reduction in size due to desiccation may in part be a consequence of a behavioral effect of pond-drying rather than a cost of accelerated development, as commonly argued in studies of plasticity to pond drying. In either case, of course, it will affect fitness later in life.

My results suggest that development rate is influenced by maternal effects, because initial mass was significantly related to age at metamorphosis. Maternal effects in amphibians are often mediated by egg size and subsequent hatchling mass, because larger eggs yield larger tadpoles (Kaplan, 1998). However, the long-term consequences of variation in maternal investment in amphibians are not well understood. For example, hatchling size in my study was related to age, but not size, at metamorphosis, even though one expects larger tadpoles to become larger metamorphs. This agrees with Loman's (2002) results. Other studies have sometimes detected the opposite situation, with maternal effects on mass but not age at metamorphosis (Sommer and Pearman, 2003), or on size and growth rate (Laugen *et al.*, 2002). Moreover, consequences of maternal effects may depend on the environment experienced by the tadpoles (Parichy and Kaplan, 1992; Laugen *et al.*, 2005). In the end, metamorphic size, age, and growth rate are highly correlated, so these distinctions might reflect small differences in the genetic architecture or history of selection in the study populations.

Population size appears to have little effect on adaptation to hydroperiod in this system. Normally, small populations have less genetic variation than large populations, which may limit their potential to adapt. However, theory also suggests that the population sizes required to appreciably reduce genetic variation in quantitative traits are very small, around 10-times lower than the sizes required to detect effects on neutral markers (Willi *et al.*, 2006). Here, I detected pretty high genetic variation both within and among populations, for small as well as for large populations (data not shown). Moreover, amphibian populations are known to be highly fluctuating (Meyer *et al.*, 1998), which reduces effective population size and may eliminate the apparent differences in size I recorded between large and small populations. Further, even if all study populations seemed relatively isolated, I cannot

exclude some gene flow between them, and this would attenuate the impact of genetic drift. An ongoing study of population genetic differentiation and landscape barriers may help to clarify these issues.

My results highlight population differences in the adaptive response to hydroperiod, although they do not clearly identify how population size or hydroperiod affect the process of adaptation. Nevertheless, there was ample variation in survival and life history among source ponds. In the end, environmental components other than hydroperiod and its temporal variation probably also affect larval life-history traits, thus diluting apparent adaptive responses to hydroperiod and making it difficult to detect “parallel” adaptation, *sensu* Kawecki and Ebert (2004). Further studies focusing on other factors that influence development or, even better, reciprocal transplant experiments are needed to disentangle the role of population size on the degree of adaptation.

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Tables

Table 1 – Mixed-effect ANOVAs for impacts of the experimental drying treatment and source population size and type on age and mass at metamorphosis (stage 42).

		age at metamorphosis		mass at metamorphosis	
Source	*d.f.**	test statistic†	*P*-value	test statistic†	*P*-value
Random effects					
Population	1	10.082	**0.005**	21.953	**< 0.0001**
Family (Population)	1	26.198	**< 0.0001**	27.385	**< 0.0001**
Fixed effects					
Treatment	1, 76	14.566	**0.0003**	152.823	**< 0.0001**
Pond type ‡	1, 12	0.572	0.4640	2.403	0.1471
Population size §	1, 12	0.598	0.4541	0.000	0.9892
Block	2, 285	7.594	**0.0006**	43.115	**< 0.0001**
Initial mass	1, 285	9.331	**0.0025**	0.713	0.3992
Treatment x type	1, 76	0.089	0.7659	1.581	0.2125
Treatment x size	1, 76	4.655	**0.0341**	1.054	0.3079
Pond type x size	1, 12	2.163	0.1671	0.078	0.7851
Treatment x type x size	1, 76	0.560	0.4568	0.681	0.4118

* *d.f.* = degrees of freedom; for fixed effects: numerator, denominator degrees of freedom

† LR-statistic for *random effects* and *F*-values for *fixed effects*

‡ in the interaction terms referred only as “type”

§ in the interaction terms referred only as “size”

Table 2 – Summary of multi-model inference to predict survival between stages 42 and 46 based on treatment, population type and size, and age and mass at stage 42. The table lists all models that fell within 2 AIC units of the best model (A) and parameter values estimated by model averaging across the six top models (B). For each model, Δ_i gives the difference from the best model in AIC_c and w_i gives the Akaike weight, which sums to 1 across all models. 95% confidence intervals (CIs) in B that do not include 0 (in bold) indicate that the parameter has an important influence on survival between stages 42-46.

A. Models within 2 AIC units of best model

Model[†]	Deviance	AIC_c	Δ_i	w_i
ty + age + mass + ty*mass	548.02	562.28	0	0.29
ty + age + mass + ty*age + ty*mass	546.58	562.91	0.64	0.21
si + ty + age + mass + ty*mass	547.29	563.62	1.34	0.15
tr + ty + age + tr*ty + ty*age	547.57	563.90	1.63	0.13
tr + ty + age + mass +ty*mass	547.96	564.29	2.02	0.11
si + ty + age + mass + ty*age + ty*mass	545.95	564.37	2.09	0.10

B. Model-averaged parameter estimates

Parameter	Coefficient	Lower CI	Upper CI
(Intercept)	-0.125	-0.621	0.370
Treatment	-0.241	-1.020	0.540
Pond type[‡]	0.369	-0.335	1.070
Population size	-0.251	-0.829	0.327
Age at stage 42	**-1.790**	**-2.580**	**-0.997**
Mass at stage 42	0.571	-0.132	1.270
Treatment * type	**1.050**	**0.205**	**1.890**
Type * age at stage 42	0.786	-0.354	1.930
Type * mass at stage 42	**-1.330**	**-2.270**	**-0.378**

[†] the following abbreviations are used: “ty” for pond type, “si” for population size, “tr” for treatment, “age” for age at stage 42, “mass” for mass at stage 42

[‡] referred to as “type” in the interaction terms

Figures

Fig. 1 – Locations of the 16 study populations in northern Switzerland. The numbers correspond to populations listed in Appendix A. Open symbols depict temporary ponds, filled symbols are permanent ponds, circles stand for small population size, and squares for large population size. The star represents the city of Zürich.

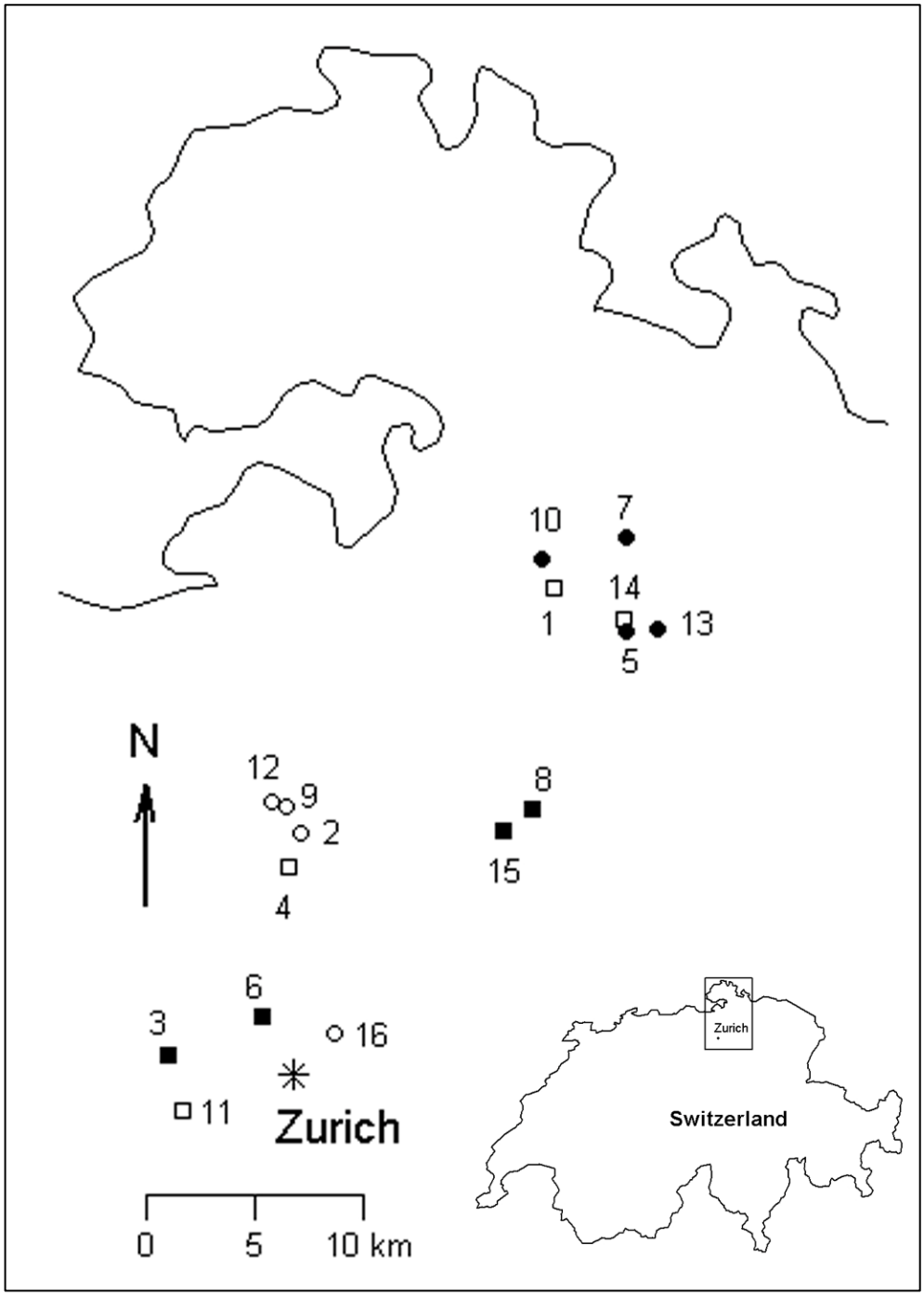


Fig. 2 – Age and mass at forelimb emergence (stage 42; left panels) and at tail resorption (stage 46; right panels) in response to water level treatments for large and small populations from permanent and temporary ponds (N = 4 populations of each type). The shape of the symbol represents population size (circle = small, square = large populations); open symbols represent temporary source ponds and filled symbols are permanent ponds. All values are population means (± SE).

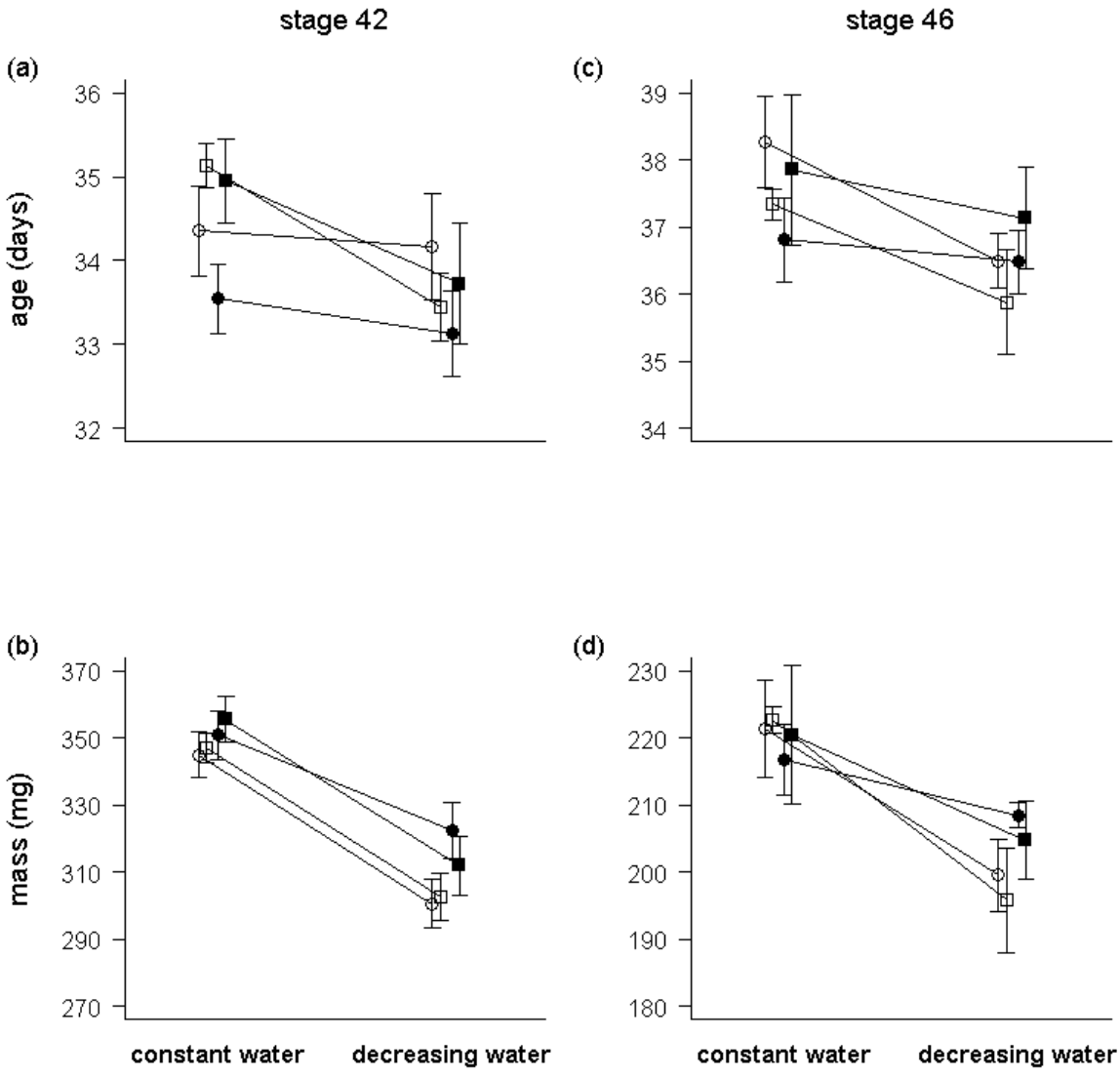
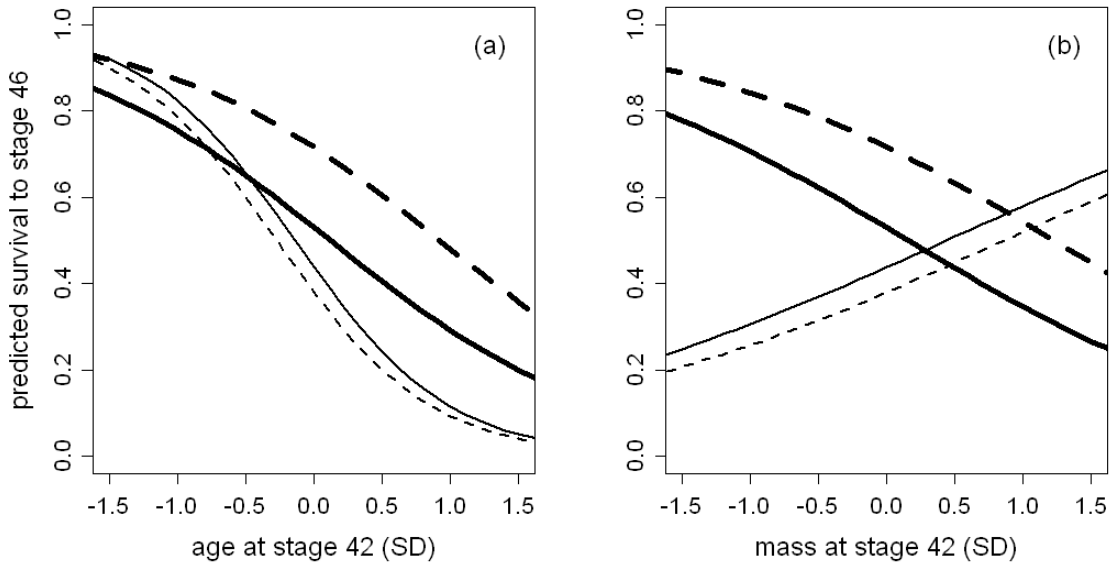


Fig. 3 – Predicted survival between stage 42 and 46 in relation to age (a) and mass (b) at stage 42 for populations from temporary and permanent ponds under two different water level treatments. Solid lines represent constant water, dashed lines are decreasing water, narrow lines are temporary ponds, and bold lines are permanent ponds.



Appendix

Appendix A – Exact locations, population sizes, and hydroperiods of the 16 *Rana temporaria* populations.

Population (Code*)	Latitude (N)	Longitude (E)	Population size†	Hydroperiod‡
Adlikon (1)	47° 34' 57"	8° 41' 58"	973 (large)	m.v. (temporary)
Allmend South (2)	47° 28' 49"	8° 32' 42"	20 (small)	-0.346 (temporary)
Anni's Pond (3)	47° 23' 17"	8° 27' 49"	1100 (large)	0.3727 (permanent)
Airport West (4)	47° 27' 60"	8° 32' 15"	1233 (large)	m.v. (temporary)
Bodenacker (5)	47° 33' 53"	8° 44' 37"	161 (small)	1.9182 (permanent)
Chäferberg (6)	47° 24' 13"	8° 31' 19"	669 (large)	m.v. (permanent)
Cold Pfarholz (7)	47° 36' 14''	8° 44' 38"	74 (small)	1.2909 (permanent)
Dättnau Lake (8)	47° 29' 23"	8° 41' 12"	3183 (large)	m.v. (permanent)
Graben (9)	47° 29' 28"	8° 32' 10"	77 (small)	0.1164 (temporary)
Hostbach (10)	47° 35' 41"	8° 41' 33"	49 (small)	m.v. (permanent)
Hueb West (11)	47° 21' 55''	8° 28' 21"	825 (large)	-0.423 (temporary)
Jonas's Weiher (12)	47° 29' 35"	8° 31' 36"	20 (small)	-0.369 (temporary)
Längeren (13)	47° 33' 56"	8° 45' 44"	105 (small)	1.9388 (permanent)
Opfiker (14)	47° 34' 11"	8° 44' 31"	805 (large)	0.2985 (temporary)
Strubikon (15)	47° 28' 51"	8° 40' 10"	1200 (large)	1.0331 (permanent)
Unterholz (16)	47° 23' 51"	8° 33' 56"	72 (small)	-2.946 (temporary)

* population codes correspond to the numbers on Fig. 1

† population size is the mean number of clutches counted during March of 3-9 years between 1997 and 2009

‡ hydroperiod is based on a PCA on maximum surface area, maximum depth, and proportion of years drying during 1997-2003; m.v. stands for missing value. Temporary ponds had a score of 0 or negative, whereas permanent ponds always had a positive score

DIVERGENCE IN *Rana temporaria* POPULATIONS DUE TO HABITAT AND POPULATION SIZE DIFFERENCES

Abstract

By comparing neutral and quantitative population differentiation in a frog (*Rana temporaria*, Linnaeus), this study aimed at detecting microevolutionary forces such as drift and selection acting within and among populations of different sizes and from different habitats. I assessed differentiation of 16 populations in relation to their size (small/large) and habitat (sunny/shady). Neutral genetic variation was evaluated using eight microsatellite loci, whereas quantitative genetic differentiation was inferred for life-history, morphological, and behavioural traits in a common-garden experiment. There was no evidence for direct phenotypic adaptation to canopy cover. However, quantitative genetic differentiation for all measured traits was higher than neutral genetic differentiation, which shows that selection is acting to promote divergence in these populations. In particular, I detected a non-random difference between Q_{ST} and F_{ST} with respect to habitat for two life-history traits and one trait reflecting the morphological shape of the tail and body. For age at metamorphosis and the shape trait, populations originating from shady habitats showed less divergence compared to populations originating from sunny habitats, suggesting different selection pressures within the two habitats. The fact that F_{ST}, Q_{ST}, and the difference between them were entirely unrelated to population size suggests a prominent role of gene flow. This is also confirmed by relatively low neutral differentiation (F_{ST} = 0.023) combined with significant isolation-by-distance. Thus, selection might not be strong enough to overcome the effects of gene flow in this system.

Introduction

Population differentiation results from the simultaneous action of three interacting evolutionary forces occurring within and among populations: gene flow, genetic drift, and natural selection (Hartl and Clark, 1997). The three processes interact strongly, and their actions are dependent on population size. Gene flow can hamper divergent selection, and its effect is larger when the recipient population is small. Genetic drift also affects small populations more strongly and leads to reduced within-population genetic diversity and enhanced among-population divergence. According to theory, natural selection leading to local adaptation should be more effective in large populations. Nevertheless, the influence of population size on the interaction between drift and selection has rarely been observed (e.g. Wade and Goodnight, 1998; Willi *et al.*, 2007). In particular, it has not been verified that, as predicted by theory, declining population size increasingly shifts the balance away from selection and toward genetic drift. A key prediction here is that selection favoring specialization should lead to local adaptation at large population size, but not at small population size.

Habitat features strongly determine the influence of natural selection. For example, in freshwater systems, forest canopy cover influences many abiotic and biotic characteristics of wetlands: closed-canopy ponds have lower temperatures and dissolved oxygen levels than open-canopy ponds, as well as lower primary productivity (Werner and Glennemeier, 1999; Skelly *et al.*, 2002). Canopy cover also strongly affects freshwater community composition (Skelly *et al.*, 1999; Schiesari, 2006), including predator abundance and diversity. All these components may greatly influence the performance of individuals, as has been shown for several amphibian species. Amphibian larvae subjected to low temperature and food availability show reduced growth rates (Laugen *et al.*, 2003), whereas higher predator risk affects

larval morphology, behavior, and growth (Van Buskirk and Relyea, 1998; Van Buskirk, 2001; Relyea, 2002). These results suggest that open- and closed-canopy habitats represent extremely different habitats; because the conditions within closed-canopy ponds are especially harsh for amphibian larvae, selection in this habitat may be stronger than in open-canopy ponds.

Comparison of divergence at neutral markers and quantitative traits provides an indirect tool to assess the strengths of natural selection and drift in shaping population differentiation (Wright, 1951; Rogers and Harpending, 1983). The neutral genetic differentiation index, F_{ST} (Wright, 1951; Nei, 1987), reflects the action of drift and gene flow. An alternative and analogous index measuring population differentiation at quantitative phenotypic traits, Q_{ST} (Rogers and Harpending, 1983; Spitze, 1993), reflects the action of drift, gene flow and selection. Therefore, because drift and gene flow act on all neutral loci and phenotypic traits equally, comparing Q_{ST} and F_{ST} provides indirect information about the power and direction of selection promoting differences among populations, as well as on which quantitative traits selection is acting. Three outcomes are possible (Merilä and Crnokrak, 2001): (1) $Q_{ST} > F_{ST}$: populations are subjected to divergent selection; (2) $Q_{ST} = F_{ST}$: the relative effects of drift and selection are indistinguishable; (3) $Q_{ST} < F_{ST}$: populations are subjected to stabilizing selection. Most studies reporting Q_{ST}-F_{ST} comparisons, as reviewed by Leinonen et al. (2008), have found higher Q_{ST} than F_{ST}, suggesting a prominent role of divergent selection in quantitative trait differentiation among populations. However, this conclusion may be simply due to the fact that most populations are geographically distant, thus experiencing very different habitat conditions. In fact, studies that focussed on neutral and quantitative divergence at small spatial scales are scarce and provide conflicting results. For example, Chapuis *et al.* (2007) and Kavanagh *et al.* (2010) found evidence for divergent selection, with

$Q_{ST} > F_{ST}$, whereas Evanno *et al.* (2006) and Rogell *et al.* (2010) found that $Q_{ST} \approx F_{ST}$ and concluded that selection was weak relative to gene flow. All four studies were conducted at spatial scales over which gene flow was judged to be feasible within a period of a few generations. General conclusions from these studies are difficult, as the number of sampled populations was usually low (except in Chapuis's study), and this is known to bias Q_{ST} estimates (O'Hara and Merilä, 2005; Goudet and Büchi, 2006).

One comparison between Q_{ST} and F_{ST} has focussed specifically on population size (Willi *et al*., 2007), and very few have explored divergence among and within distinct habitats (e.g. Chapuis *et al.*, 2007; von Wettberg *et al.*, 2008). No study has compared population size and different habitats together at small spatial scales. To fill this knowledge gap, I assessed neutral and quantitative population divergence with respect to population size and habitat type in 16 populations of a frog, *Rana temporaria* (Linnaeus), occurring in a small area. Neutral differentiation was evaluated with microsatellites, and quantitative differentiation was inferred for various phenotypic traits measured in tadpoles raised in a common-garden experiment. The final outcome can be viewed as an initial step toward estimating the relative importance of selection, drift, and gene flow within a collection of populations. In particular, the aims of this study were two-fold: first, I wanted to explore the association between neutral and quantitative divergence and habitat divergence, and how this association is modified by population size. Second, I wanted to know which traits are potentially under selection.

A key expectation was that small populations would be more divergent from one another than large populations due to higher drift, both neutrally and quantitatively and irrespective of the habitat of origin. Also, I hypothesized enhanced population divergence between populations occupying different canopy habitats. Additionally, if

there is stronger selection within shady ponds than sunny ponds, as outlined above, populations in closed canopy sites should display reduced among-population differentiation than populations in open habitats. All the quantitative traits I studied, related to larval morphology, behaviour, and life history, were potentially subjected to selection driven by habitat features, but to different extents. In fact, response to selection depends on the genetic architecture of the traits (i.e. number of genes involved and proportion of phenotypic variation due to additive genetic effects) and how the traits are related to fitness (Merilä and Sheldon, 1999). Thus, morphological traits are expected to respond faster to selection due to their higher additive genetic variance and heritability, but life-history-traits to be more strongly exposed to selection, as their relationship to fitness is relatively strong.

Material & Methods

Study species and populations

Rana temporaria (Linnaeus) is a common amphibian in Europe, with a very broad habitat range. It breeds in very different water bodies that are located in both open and forested landscapes (Nöllert and Nöllert, 1992). Adults and juveniles are mostly terrestrial. Although the annual migrations to and from breeding sites may extend over hundreds of meters or a few kilometers, most juveniles and adults are philopatric (Seitz *et al.*, 1992; Vos *et al.*, 2007; Kovar *et al.*, 2009).

In March 2008 I collected eggs from 16 populations in northeastern Switzerland within a total area of approximately 750 km^2 (Fig. 1; exact locations and details are given in Appendix A). The populations were selected to create a 2-by-2 factorial design, with small and large population sizes crossed with sunny and shady habitats (four replicates of each combination). Population size was estimated from the harmonic mean across years of clutch counts made just after oviposition during

March 1997-2008. The harmonic mean was used because it reflects the genetic effective population size when populations fluctuate in size (Frankham *et al.*, 2004, p. 63). Small populations ranged from 16 to 158 egg clutches (arithmetic mean = 65), and large populations from 582 to 1'241 (mean = 829). Pond canopy cover was estimated by means of hemispherical photographs taken in April and June 2008 (Evans and Coombe, 1959; Anderson, 1964). Canopy cover was defined as the fraction of area obstructed by leaves within a strip running across the image that represented the daily passage of the sun, and was calculated as the mean of these two surveys. Sunny ponds had either no or little canopy cover (mean = 0.09), and shady ponds ranged from 33 to 64% cover (mean = 0.49). There was no spatial autocorrelation in population size or habitat: Mantel tests revealed non-significant associations between log-transformed geographical distance and population size or habitat expressed as categorical factors (population size: $r = 0.073$, $p = 0.214$; habitat: $r = 0.025$, $p = 0.388$).

I collected seven freshly laid *Rana temporaria* egg clutches from each population for use in the common garden experiment. The clutches were held separately in outdoor tanks until the experiment began, when the larvae were about 3-4 days old (stage 24; Gosner, 1960). Additionally, I sampled single eggs from up to 42 clutches in each population for analyses of neutral marker variation. In the smallest populations I sampled from all available clutches, and in larger populations I collected samples from different parts of the pond to reduce the chance of including multiple clutches sired by the same male. Sample sizes are reported in Appendix C. After hatching, tadpoles were preserved in alcohol at -20°C until DNA extraction.

Genetic analyses

To estimate neutral population differentiation (F_{ST}), allelic variation was scored at 8 microsatellite loci: Rtempμ4, Rtempμ7 (Rowe and Beebee, 2001), RtμR, RtμB, RtμP (Pidancier *et al.*, 2002), Rt2Ca2-22, Rt2Ca30 (Teacher *et al.*, 2009) and RtSB14 (Berlin *et al.*, 2000). DNA was extracted from tadpole tails with the BioSprint 96 workstation and the BioSprint 96 DNA Blood Kit from Qiagen (www.qiagen.com). A few samples were extracted manually with the QIAamp DNA Mini Kit. Polymerase chain reaction (PCR) amplifications were performed with1 μl DNA sample, 4 μl of a mix of the primers (from Applied Biosystems and Microsynth) and Taq polymerase (from Qiagen Multiplex PCR Kit). PCR cyclings consisted of 15 minutes at 95°C for the initial denaturation, followed by 30 cycles of 30 s at 94°C, 90 s on 59°C (annealing steps), 60 s at 72°C and 30 s at 60°C. Thereafter, I added 20 μl of LIZ-HiDi solution to 1.6 μl of each PCR product. After denaturating these final solutions for 2 minutes at 95°C, I processed them with an ABI 3730 sequencer.

Alleles were scored with GeneMapper Software (version 3.7, Applied Biosystems). Reactions with unclear or vague genotypes were repeated, both in multiplex and singleplex. I evaluated the frequency of genotyping errors by repeating 90 randomly-selected samples (corresponding to about 15% of the samples) and estimating the error rate per allele (calculated as the ratio of observed incorrect alleles over the total number of allelic comparisons) and per reaction (incorrect genotypes divided by the total number of reactions). These rates were then summarized both for each locus and across all loci (Bonin *et al.*, 2004; Hoffman and Amos, 2005). This allowed me to detect odd genotypes, systematic errors or problematic markers. The final error rate, calculated over all 8 microsatellite loci, improved from 0.017 to 0.004 per allelic comparison, and from 0.023 to 0.004 per reaction, after rechecking all data and correcting some scoring errors.

The microsatellite data were first tested for null alleles, stutters or large allele dropouts using MICRO-CHECKER version 2.2.3 (VanOosterhout *et al.*, 2004; freely downloadable from http://www.microchecker.hull.ac.uk). The confidence interval for the Monte Carlo simulations was set to 95%, with 1'000 runs. Samples where stuttering was detected were rechecked in GeneMapper. Selective neutrality was tested with FDIST2 (Beaumont and Nichols, 1996; software freely available at www.rubic.rdg.ac.uk/~mab/software.html), which generates an expected F_{ST}-distribution as a function of observed heterozygosity under the Island Model (Wright, 1951). In the end, the loci Rt2Ca30, RtSB14 and RtμB were discarded from further analysis. The former had a null allele frequency of 20-40% in every population (based on Brookfield Estimator 2; Brookfield, 1996) and the two latter markers had observed F_{ST} values below the 95% confidence intervals of the expected distribution (therefore indicating homogenizing selection; Appendix B). Consequently, I used five loci to calculate pairwise F_{ST}-estimates, with FSTAT 2.9.3.2 (Goudet, 1995) from variance components of genotype frequencies according to Weir & Cockerham (1984).

Mean number of alleles, allelic richness (R_S, El Mousadik & Petit, 1996), gene diversity (H_S, Nei, 1987), and the inbreeding coefficient F_{IS} per sampled population were assessed with FSTAT 2.9.3.2 (Goudet, 1995), and observed and expected heterozygosity (H_o and H_e) with ARLEQUIN version 3.1 (Appendix C). I observed no deviations from Hardy-Weinberg equilibrium for 10 of 16 populations assessed with GENEPOP version 4.0.10 (Raymond and Rousset, 1995) by the Markov Chain method (Appendix C). Linkage disequilibrium was tested with the log-likelihood G-statistics implemented in FSTAT 2.9.3.2 (Goudet, 1995) after Bonferroni corrections. Loci RtμP and Rtempμ7 were linked in two populations.

Common-garden experiment

In order to estimate population differentiation in quantitative traits (Q_{ST}), I performed a common-garden experiment in an outdoor field at Irchel Campus, University of Zürich. The experiment had a 2 x 2 factorial block design, with population size (small/large) and population habitat (sunny/shady) as factors. There were four source populations for every combination of population size and habitat, seven families for each population, and two replicates; this yielded a total of 224 experimental units.

In late February 2008 I filled 80 l tanks with water, and at the beginning of March I put in each tank 40 g dried beech leaves (*Fagus sylvatica*), zooplankton collected from a nearby pond, and 2 g of rabbit food, in order to create semi-natural conditions and provide some food for the tadpoles for the duration of the experiment.

I started the experiment on 1 April 2008, when I counted out three sets of 25 tadpoles from each of 7 families for every population. Two of these sets were introduced to the experiment, giving 25 tadpoles in every mesocosm. The third set of tadpoles, chosen haphazardly, was preserved in formaldehyde in order to assess tadpole mass at the beginning of the experiment. All tadpoles were kept in a small box, which floated in its assigned mesocosm until all tadpoles were sorted, at which point tadpoles were released from the box. The density of tadpoles in the tanks was decreased during the experiment because animals were removed periodically for ancillary experiments. In total, 14 tadpoles were removed from each tub by day 35; the remaining 11 were left to develop until metamorphosis.

On 27 April I measured tadpole behaviour. This consisted of five visits to each tank during the day, between 10:00 and 16:00, on which I recorded the number of visible tadpoles and their activity (swimming, feeding or resting). From these data I calculated the proportion of visible tadpoles that were active (swimming or feeding divided by all visible tadpoles) and the proportion of tadpoles that were hiding (1 –

[number visible/estimated number alive]). The estimated number alive was the total number of tadpoles that reached metamorphosis; this assumes that most mortality occurred early in the larval period.

On 6-8 May I measured the external morphology and mass of 5 tadpoles per tank. Tadpoles were lightly anaesthetized, photographed in side and bottom views, weighed, and then returned to their tanks. I later digitized the pictures using image analysis software (Rasband, 1997-2011), assigning 22 landmarks in the side view and 13 in the bottom view. The landmarks are defined in Appendix D. Morphological shape was then assessed in two different ways: as size-corrected lengths of distances between specific landmarks, and as geometric morphometric measures of shape (Bookstein, 1990). Geometric morphometric analyses were done with PCAGen6p version 16.07.2002 from the IMP Integrated Morphometrics Package (freely available from www.canisius.edu/~sheets/morphsoft.html). I subsequently retained the number of relative warps required to explain at least 80% of the total shape variation observed.

Beginning on 20 May the tanks were checked daily to remove tadpoles at stage 42 (forelimb emergence). Metamorphs were held in small boxes with a little bit of water until they reached stage 46 (complete tail resorption) and were then weighed. Those that died between stages 42 and 46 were scored as survivors with missing values for mass. All surviving froglets were released into their pond of origin.

In summary, I recorded six life-history traits (initial mass, mass at day 36, early growth rate – i.e. [(mass at day 36 – initial mass) / 36] – age and mass at metamorphosis, and survival), nine morphological traits (head length, head depth, head width, mouth width, tail length, maximum tail fin depth, tail depth at half the length of the tail, tail muscle depth at ½, and tail muscle width at the base), two relative warps for the bottom view (bottom RW1, explaining 45% of the variation, and

bottom RW2, 37%), five relative warps for the side view (side RW1, 33%; side RW2, 24%; side RW3, 13%; side RW4, 7%; side RW5, 5%), and two behavioural traits (i.e. proportions of active tadpoles and hiding tadpoles).

Statistical analysis

I first checked for phenotypic differences associated with population size and habitat, using mixed-effects models. Spatial block, population size and habitat were fixed factors, and populations and families nested within population were random factors. In addition, in the models for life-history and morphological traits, I tested the importance of three covariates together. The covariates were initial mass, mass at day 36, and tadpole density at the end of the experiment to account for body size differences. These tests revealed that tank density affected mass at metamorphosis, whereas initial mass was important for mass at day 36, early growth rate and age at metamorphosis. Morphological traits were significantly related to mass at day 36 and in some cases also initial mass. The responses expressed as proportions (i.e. survival, active and hiding tadpoles) were arc-sine-square root transformed to meet the assumption of normality of the data.

Within and between population variance components were estimated for each pairwise population comparison and for each single trait. The estimates came from mixed-effects models including block as a fixed effect, and population and family nested within population as random factors. Covariates that were significant in the previous analyses were also included in these models. Q_{ST}-estimates were calculated using the formula $Q_{ST} = V_B / (V_B + 2*V_W)$, where V_B stands for the variance component between populations and V_W for variance among families within population. I also calculated overall mean Q_{ST}'s for traits categorized into four groups: life-history, geometric morphometric measures, size-corrected lengths, and

behavior. This was done by standardizing the variance components of the individual traits in order to equalize the influence of traits on the overall mean Q_{ST}. This involved dividing family and population variances by their total variance (including the residual variance), and then using the formula for Q_{ST} given above. For the overall Q_{ST} for life-history traits I excluded initial mass because it is known to be heavily affected by maternal and environmental effects (Kaplan, 1998).

Two-sided partial Mantel tests (999 permutations) were used to compare the matrices of the pairwise comparisons and determine whether neutral and quantitative genetic divergence were associated with both habitat differences and population size differences, in each case accounting for the other. Population size was expressed as dissimilarity matrix with three possible values. Because divergence should be largest in small populations due to drift, intermediate in comparisons between different population size, and smallest between large populations, I assigned -1 to comparisons between small populations, 1 for large populations and an intermediate value of 0 for comparisons between populations of different size. The habitat was first expressed as a similarity matrix with a value of 0 for pairs of same habitat, and 1 for pairs involving comparisons between different habitats, as I expected comparisons across habitats to show the greatest divergence. As I did not find any significant result with the similarity matrix, I expressed an alternative hypothesis with a dissimilarity matrix. This contained values of -1 for pairs of sunny ponds, 1 for pairs of shady ponds, and an intermediate value of 0 for comparisons between populations from different habitats. The idea here was that closed-canopy imposes strong selection for traits associated with rapid growth and efficient resource consumption, so that tadpoles from all closed-canopy sites should exhibit similar traits. Sunny ponds represent instead a diverse range of habitats.

I began by testing whether F_{ST} and Q_{ST} estimates were themselves correlated to population size, habitat, and geographic distance. For F_{ST}, I expected a relationship with population size because small populations are affected more strongly by genetic drift, but no relationship with habitat because F_{ST} is a measure of neutral divergence that is not affected by loci subjected to selection. I tested for isolation by distance following Rousset (1997) between the matrices of pairwise $F_{ST} / (1 - F_{ST})$ and pairwise log transformed distances. A positive result would imply that more distant populations are connected less frequently by gene flow and therefore express more neutral genetic differentiation due to drift. The corresponding analyses of Q_{ST} evaluate whether quantitative differentiation is related to the canopy gradient or population size, and test whether quantitative traits are drifting apart due to distance much the way neutral markers do.

Subsequently, to disentangle the effects of selection and drift on quantitative population divergence, I used a matrix depicting pairwise Q_{ST}-F_{ST} differences, which expresses quantitative divergence after accounting for neutral divergence. As before, I checked it with the matrices of habitat and population size dissimilarity described earlier. Here, significant results imply that selection is driving population divergence. The expectations in respect of habitat are described previously. In respect to population size, drift would especially affect small populations, leading to smaller Q_{ST}-F_{ST} difference for comparisons between populations of small size. The Q_{ST}-F_{ST} difference is expected to increase with population size, as drift becomes smaller and selection more effective. In a further step I evaluated the influence of distance on the results, because F_{ST} was highly correlated with distance.

Statistical tests were conducted in R 2.13.2 (R Development Core Team, 2010), with the packages “nlme” (for mixed-model ANOVAs, method REML: Pinheiro *et al.*,

2009), “ncf” (for two-sided Mantel tests: Bjornstad, 2009), and “boot” (for calculating CIs for Q_{ST} values: Davison and Hinkley, 1997; Canty and Ripley, 2011).

Results

Genetic analyses

I scored 568 individuals at 5 loci, with an average of 35.6 individuals per population. The number of alleles sampled per locus ranged from 2 to 29 (average 11.8), and overall allelic richness ranged from 2 to 9.78 (average 4.91). Allelic richness (R_S) per population ranged from 4.295 to 5.131 (average 4.713), gene diversity (H_S) from 0.564 to 0.680 (average 0.624), observed heterozygosity (H_o) from 0.525 to 0.657 (average 0.576), inbreeding coefficient (F_{IS}) from -0.066 to 0.183 (average 0.075; see Appendix C for single population values). Pairwise F_{ST} values ranged from -0.004 to 0.076 (Appendix E), and the global F_{ST} was 0.023 (± 0.004 SE; calculated by jackknifing over loci) or 0.024 (95% CI: 0.017-0.031; calculated by bootstrapping). There was no difference between large and small populations in observed heterozygosity (H_o), inbreeding coefficient (F_{IS}) or fixation index (F_{ST}, for small populations 0.015, large 0.029) (randomization tests based on 2’000 permutations: all $p > 0.15$). Also, I detected no effect of population size on pairwise F_{ST} values (Mantel test: $r = 0.171$, $p = 0.197$; after correcting for distance, partial Mantel test: $r = 0.156$, $p = 0.219$). Habitat had no influence on F_{ST} in two kinds of tests: global values did not differ between shady and sunny ponds (0.027 and 0.020, respectively; $p = 0.748$) and comparison of F_{ST} with habitat differences revealed no association (Mantel test: $r = -0.060$, $p = 0.376$; partial Mantel test correcting for distance: $r = -0.082$, $p = 0.346$). There was highly significant isolation by distance at neutral markers (Mantel test: $r = 0.4705$, $p = 0.001$).

Phenotypic divergence

There was no significant difference in any phenotypic trait between populations of different sizes or from different habitats. Most traits showed significant variation among families nested within population, whereas variation among populations was present in less than half the traits (Appendix F). Some of the life-history traits, such as mass at day 36 and early growth rate, varied significantly among populations, as did both the behavioural traits and two of the seven relative warps.

Quantitative trait divergence

Table 1 contains results of all Mantel tests evaluating quantitative genetic divergence. I found significant correlations due to habitat for two of the six life-history traits, and these were in opposite directions. For initial mass, Q_{ST} was larger among shady than sunny ponds (mean = 0.299 [95% CI: 0.217-0.308] versus 0.078 [0.071-0.153], respectively; partial Mantel test: r = 0.324, p = 0.038). For age at metamorphosis, shady ponds had the lowest Q_{ST} and sunny ponds the highest (Fig.2; 0.061 [0.053-0.144] versus 0.235 [0.171-0.263], respectively; r = -0.281, p = 0.040). This pattern was due to relatively large among-family variances and small among-population variances in shady ponds, small among-family variances and large among-populations variances in sunny ponds, and intermediate values for comparisons between different habitats (Fig. 3). For both initial mass and age at metamorphosis, comparisons between different habitats resulted in intermediate values of Q_{ST} (initial mass: 0.178 [0.073-0.307]; age at metamorphosis: 0.144 [0.026-0.315]). Further, an effect of habitat was also found on one morphological trait (bottom RW1: r = -0.455, p = 0.01). In this case, Q_{ST} was the largest in sunny habitats (0.767 [0.612-0.755]), and the smallest in shady (0.277 [0.292-0.443]). The very high value of Q_{ST} in the sunny habitat was caused by an extremely small

among-family variance. In respect to population size, I found no relationship, with the exception of one morphological trait (tail depth at ½: r = 0.394, p = 0.013). In this single case, there was nearly no among-population variance within small populations, and this yielded an extremely small value of Q_{ST} (0.005 [0.004-0.059] among small populations and 0.214 [0.129-0.217] among large populations).

No correlation was found on overall Q_{ST}s for trait classes, neither due to habitat nor to size. Accounting for geographic distance in these tests did not affect the results.

Quantitative differentiation was positively correlated with geographic distance for two traits, size-corrected head width (r = 0.184, p = 0.026) and tadpole activity (r = 0.192, p = 0.017). None of the trait classes had Q_{ST} values associated with distance.

F_{ST}-Q_{ST} comparisons

Quantitative differentiation for all traits was higher than neutral differentiation. The lowest Q_{ST} was 0.087 [0.076-0.102] for tail depth at ½; the highest Q_{ST} was 0.531 [0.505-0.556] for bottom RW1. As reported earlier, F_{ST} was much smaller than any of these values (0.017-0.031 95% CI).

The significant effect of habitat found for the two life-history traits and one morphological trait persisted also after taking neutral variation into account. Habitat affected the Q_{ST}-F_{ST} difference on initial mass (r = 0.320, p = 0.047), age at metamorphosis (r= -0.283, p = 0.037), and bottom RW1 (r = -0.457, p = 0.008). Again, the Q_{ST}-F_{ST} relationships in life-history traits were in opposite directions: for age at metamorphosis Q_{ST} values were especially low relative to F_{ST} in shady ponds compared to sunny ponds (Fig. 2), as it was for the morphological trait. The opposite was true for initial mass.

When taking neutral variation into account, the population size effect remained also for the single morphological trait (tail depth at ½: r = 0.380, p = 0.020). The Q_{ST}-F_{ST} relationships were higher than randomly expected in large populations compared to small populations. As for quantitative differentiation, taking distance into account did not change the results.

Discussion

By comparing neutral and quantitative population differentiation, this study aimed at detecting microevolutionary forces such as drift and selection acting within and among populations of different sizes and from different habitats. My study revealed three key results. First, I obtained no evidence for direct phenotypic adaptation to canopy cover, as there was no systematic difference in quantitative traits between habitats. Second, quantitative genetic differentiation for all measured traits was higher than neutral genetic differentiation, which shows that selection is acting to promote divergence in these populations. Some variation in quantitative divergence was related to canopy cover. Third, F_{ST}, Q_{ST}, and the difference between them were entirely unrelated to population size. In the following, these main findings are discussed. I will also present some general thoughts on criticisms about $Q_{ST} – F_{ST}$ comparisons and the potential biases underlying the approach.

The lack of phenotypic divergence between open- and closed-canopy sites indicates that *R. temporaria* have not adapted to the canopy gradient. This was unexpected, because I examined many traits and had reasonably good statistical power to detect adaptation (8 populations for each habitat, and 7 families within each population). Moreover, the high among-family variation in most traits suggests that these populations have the capacity to respond to natural selection. On the other hand, low levels of population divergence point toward the pervasive importance of

gene flow, and in fact many of my ponds are within a few kilometres of each other. However, many other studies have discovered local adaptation on spatial scales that are fine relative to the scale of dispersal, presumably because selection is sufficiently strong to overwhelming the homogenizing effect of gene flow (e.g. Gomez-Mestre and Tejedo, 2004; Kavanagh *et al.*, 2010; Junge *et al.*, 2011). Some of these studies even note phenotypic differences in amphibians with respect to canopy cover at relative small spatial scale. Van Buskirk and Arioli (2005),working on the same species in nearly the same area as I did, tested in a common-garden experiment populations originating from ponds that encompassed three different environmental gradients (canopy cover, pond hydroperiod and predation risk). Their average tadpole phenotypes were often correlated with habitat features of the source ponds, but mainly to predation risk. A response to canopy cover was detected on all morphological traits. The fact that individuals from sunny ponds were morphologically similar to predator-induced tadpoles suggests that their response might be also due to predation risk, rather than merely canopy, as evidenced by the negative correlation between these two factors. Thus, diverse environmental gradients may act interactively, and the extent of the evolutionary response will finally depend on their combination. For instance, closed-canopy cover and high predation risk may both select for rapid growth rates, but no adaptation may occur in shady ponds with few predators because selection is acting in opposite directions. Still, Skelly (2004) found faster embryonic development in individuals originating from shady ponds, even within a set of 13 wetlands sampled over an area as small as 12 km^2. However, embryonic development is highly influenced by maternal rather than genetic effects. Even if maternal effects may play meaningful a role in adaptation (Laugen *et al.*, 2002; Räsänen *et al.*, 2003), their importance here makes Skelly's results difficult to interpret as local adaptation. Moreover, Skelly reported no divergence in post-

hatching growth or development, which suggests that these have not diverged in his study populations. Nevertheless, the Skelly (2004) and Van Buskirk and Arioli (2005) studies highlight that adaptation over very small spatial scales is possible.

Other studies on canopy gradients involved comparisons between open-canopy specialists and canopy generalist species. Growth rate and survival are generally higher when tadpoles grow in sunny ponds, but generalists sometimes perform well in shady ponds as well, while specialist species do very poorly in the shade (field experiments: Werner and Glennemeier, 1999; Skelly *et al.*, 2002). The same results were found in a common-garden set-up involving canopy treatments: the generalist species had shorter larval period than open-canopy specialist in closed-canopy treatments (Skelly *et al.*, 2002). However, conclusions from these two last studies are hard to infer, because these experiments have measured traits in the field, or in a common-garden set-up with benthic substrate and water from closed- or open-canopy ponds, thus testing abiotic and biotic conditions rather than really detecting genetic differences due to pond origin. Nevertheless, these studies indicate higher performance over habitats for the generalist species, which might also imply a role of phenotypic plasticity in traits related to performance in the sun and shade. Adaptive phenotypic plasticity is quite common in the generalist *R. temporaria*, and it will be favoured by gene flow. As demonstrated by Lind *et al.* (Lind and Johansson, 2007; Lind *et al.*, 2011), gene flow and habitat heterogeneity in pond drying regimes were responsible for evolution of plasticity. If the outcome of selection in my target populations is phenotypic plasticity, this might explain why I did not detect any difference in quantitative traits of larvae raised in the common-garden experiment. In fact, in this case there will be strong environment-by-genotype interactions, and trait differentiation will not be detected in a common-garden set-up (Whitlock, 2008).

I did find higher quantitative genetic differentiation than neutral genetic differentiation for all measured traits, which indicate that selection is acting to favour divergence in these populations. Thus, selection might be not strong enough to overcome the effects of gene flow. There was some evidence that the difference between Q_{ST} and F_{ST} was non-random with respect to the canopy habitat of the pond. In particular, I observed low levels of divergence for age at metamorphosis and bottom RW1 among populations from shady ponds compared to populations from sunny ponds. This may indicate that selection on these traits was more uniform within the shady habitat and more heterogeneous in the sunny habitat, whose populations showed high levels of divergence. This idea was suggested by Porcher *et al.* (2004), who found that Q_{ST} values increased with increasing selection heterogeneity. In amphibians, Relyea (2002) detected significant differences in two life-history traits among four open-canopy populations, but not among four closed-canopy pond populations. Although he did not actually test for divergence between habitats, or for different levels of variation between habitats, Relyea asserted that sunny ponds were more different from one another. Moreover, he observed that shady ponds have consistent biotic conditions, whereas sunny ponds are highly variable in respect to competition and predator composition, at least in his study area. This confirms the idea that shady ponds are subjected to uniform selection, whereas sunny ponds experience more heterogeneous selection.

I found no effect of habitat in classes of related traits, suggesting that the balance between selection and drift is specific to individual traits (McKay and Latta, 2002). In fact, each trait may be under a different selection regime, so that Q_{ST} values for different traits may be very different. Within life-history traits, mass at metamorphosis produced a similar Q_{ST} pattern (data not shown) as age at metamorphosis. Even if the differences in Q_{ST} values for mass at metamorphosis were not significant, this

suggests that habitat is acting in the same direction on at least these two traits. Otherwise, if canopy cover would exert selection exclusively on larval development, the effect on mass might be due rather to a correlated response, as age and mass at metamorphosis in amphibians are closely associated (e.g. Travis, 1980).

The difference between Q_{ST} and F_{ST} was entirely unrelated to population size except for one trait. This suggests that either adaptive divergence is unaffected by population size within the range studied here, or the population counts used in this study do not accurately reflect genetic effective population size. I am more inclined towards the second explanation, as the very low overall F_{ST} values combined with significant isolation-by-distance clearly show that gene flow occurs regularly among sites within about 10 km of one another and is infrequent at larger scales. This suggests that genetic drift cannot shape population differentiation at the scale of a few km, but that drift might be detectable over the scale of my entire study area. The fact that population size was unrelated to F_{ST} confirms the dominant role of gene flow in this system. However, neutral genetic variation may reflect recent past rather than present history, and my demographic data include only a few generations. Additionally, the levels of population size that cause erosion of additive genetic variation are around 10 times lower than those that cause erosion in neutral marker diversity (Willi *et al.*, 2006). Thus, in this context, maybe my populations are just not small enough.

My use of a full-sib common garden experiment potentially introduces two sources of bias. First, the breeding design confounds purely additive genetic effects with non-additive variance. Q_{ST} estimates based on broad-sense genetic variation may be biased by epistasis and dominance, and this bias should be stronger for life-history traits than morphological traits. If non-additive effects lead to an overestimation of the within-population variance, this would cause an

underestimation of Q_{ST}. Therefore, some have suggested that a likely signature of important levels of dominance and epistasis is a pattern of $Q_{ST} < F_{ST}$ (Toro and Caballero, 2005; Goudet and Büchi, 2006). Whitlock (2008) argues that in the case of Q_{ST} estimates biased by non-additive effects, evidence for diversifying selection would be conservative and inadequate for stabilizing selection. In my study, Q_{ST} was always greater than F_{ST}, suggesting that any bias from non-additive effects was too weak to overcome effects of divergent selection.

A second source of bias comes from maternal and environmental effects. The first-generation common-garden approach greatly reduces the influence of the environment on tadpole phenotypes, but does not eliminate carry-over effects of the environment in which parents developed as larvae and adults. Indeed, it was for this reason that I discarded initial mass from analyses of overall Q_{ST}, because this trait is known to be affected by maternal and environmental components (Kaplan, 1998). Moreover, I included initial mass and early growth as covariates in many of my models to mitigate as far as possible maternal effects on traits later in the larval period. Maternal effects could be similar to epistasis and dominance in causing an overestimation of the within-population variance component (Whitlock, 2008), or they could inflate variation among ponds if adults in different ponds experience very different conditions (Merilä and Crnokrak, 2001). In the end, I suspect that these sources of bias are not especially large, partly because Leinonen *et al.*'s (2008) meta-analysis of many published data sets revealed no systematic difference in Q_{ST} estimates between full-sib or half-sib breeding designs.

The use of F_{ST}-Q_{ST} comparisons has been criticized on both conceptual and methodological grounds. There is a lively debate underway regarding the best way to measure neutral population differentiation (e.g. Jost, 2008; Gerlach *et al.*, 2010; Leng and Zhang, 2011; Meirmans and Hedrick, 2011), and much discussion of the

precision and biases in estimating quantitative population differentiation (e.g. Lopez-Fanjul *et al.*, 2003; O'Hara and Merilä, 2005; Goudet and Büchi, 2006). However, I argue that comparative studies of Q_{ST} and F_{ST} are valid as an exploratory analysis in order to see whether there is potential for selection favoring adaptation to particular environmental features and to identify which traits are selected. The number of populations sampled should be high (O'Hara and Merilä, 2005), and possible artefacts due to isolation-by-distance in quantitative traits should be controlled for. Most of all, studies such as this should move beyond the phenomenological level of estimating divergence to test putative agents of selection, as was recently done by Hangartner *et al.* (2011). Isolation-by-distance is commonly tested for neutral differentiation, but not for quantitative divergence, or at least is rarely reported. Moreover, it is not especially informative to use Q_{ST}-F_{ST} comparisons to explore populations that are already known to be divergent in quantitative traits. In this case, I encourage scientists to confirm local adaptation with transplant experiments or common-garden experiments that incorporate the suspected selective agents as treatments. Additionally, as Q_{ST}-F_{ST} studies are best used as exploratory analyses, I encourage researchers to investigate as many candidate traits as possible, and to publish non-significant results along with the significant. This will support our efforts to achieve a general synthetic understanding of the extent and targets of population divergence, both adaptive and otherwise.

This study reveals diversifying selection on two life-history traits and a morphological trait due to habitat differences in canopy cover, with stronger selection in shady ponds and more heterogeneity in selection within sunny ponds. High levels of gene flow among these populations make any inference about drift, selection and population size problematic. High gene flow also indicates that adaptive phenotypic plasticity induced by canopy cover may be an important part of the process of local

adaptation. A next step in the direction of disentangling the forces of genetic drift and selection on small populations will require better-isolated populations, whereas the potential importance of phenotypic plasticity will require experiments testing environment-by-genotype interactions, thus comparing populations in a common-garden experiment with multiple treatments representing canopy cover or other relevant habitat gradients.

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Tables

Table 1 Results for the partial Mantel tests performed for correlations between Q_{ST}s for trait classes and their single traits, as well as for Q_{ST}–F_{ST} differences (dependent matrices), and habitat and population size differences. The correlation coefficient $r_{12.3}$ gives the correlation between the dependent matrix with habitat after correcting for population size, whereas $r_{13.2}$ of population size after correcting for habitat. Boldface highlights significant results.

Tests:	Q_{ST} ~ habitat + population size				(Q_{ST} – F_{ST}) ~ habitat + population size			
	$r_{12.3}$	p	$r_{13.2}$	p	$r_{12.3}$	p	$r_{13.2}$	p
Life-history traits	-0.026	0.430	0.146	0.195	-0.034	0.434	0.114	0.277
Initial mass	**0.324**	**0.038**	-0.061	0.348	**0.320**	**0.047**	-0.075	0.336
Mass at day 36	0.084	0.315	0.243	0.120	0.077	0.342	0.225	0.112
Early growth rate (days 0-36)	0.098	0.302	0.256	0.093	0.091	0.316	0.237	0.127
Mass at metamorphosis	-0.238	0.130	-0.115	0.299	-0.240	0.123	-0.124	0.272
Age at metamorphosis	**-0.281**	**0.040**	0.050	0.439	**-0.283**	**0.037**	0.038	0.422
Survival	0.206	0.145	-0.293	0.088	0.200	0.181	-0.297	0.081

Tests:	Q_{ST} ~ habitat + population size				(Q_{ST} – F_{ST}) ~ habitat + population size			
	$r_{12.3}$	p	$r_{13.2}$	p	$r_{12.3}$	p	$r_{13.2}$	p
Morphometric traits (shapes)	-0.079	0.333	-0.064	0.413	-0.090	0.331	-0.090	0.329
Bottom RW1	**-0.459**	**0.009**	-0.155	0.234	**-0.457**	**0.008**	-0.163	0.212
Bottom RW2	-0.021	0.450	0.069	0.371	-0.024	0.463	0.060	0.414
Side RW1	0.276	0.130	0.200	0.233	0.279	0.136	0.191	0.268
Side RW2	0.230	0.146	0.035	0.447	0.228	0.141	0.026	0.436
Side RW3	-0.086	0.358	-0.039	0.416	-0.089	0.349	-0.052	0.403
Side RW4	-0.179	0.165	-0.025	0.453	-0.184	0.161	-0.036	0.428
Side RW5	-0.121	0.283	-0.192	0.198	-0.127	0.290	-0.204	0.155
Morphology traits (lengths)	-0.150	0.290	0.323	0.057	-0.162	0.281	0.298	0.081
Head length	0.076	0.352	0.303	0.065	0.071	0.380	0.290	0.068
Head depth	-0.157	0.162	-0.112	0.238	-0.162	0.176	-0.124	0.237
Head width	-0.249	0.106	-0.142	0.248	-0.255	0.083	-0.153	0.220
Mouth width	-0.128	0.245	0.115	0.274	-0.133	0.245	0.105	0.275
Tail length	-0.056	0.426	0.240	0.084	-0.060	0.363	0.233	0.095
Maximum tail depth	-0.233	0.192	0.102	0.452	-0.238	0.195	0.088	0.467
Tail depth at ½	-0.230	0.194	**0.394**	**0.013**	-0.237	0.184	**0.380**	**0.020**
Tail muscle depth at ½	-0.070	0.354	0.218	0.094	-0.075	0.338	0.205	0.091
Tail muscle width at the base	-0.122	0.243	0.187	0.153	-0.126	0.229	0.179	0.150
Behaviour	0.106	0.319	0.090	0.348	0.104	0.311	0.080	0.365
Active tadpoles	0.178	0.210	0.212	0.166	0.176	0.229	0.203	0.204
Hiding tadpoles	0.093	0.336	-0.106	0.335	0.091	0.338	-0.116	0.304

Figures

Fig. 1 – Map showing the locations of the 16 study populations in northern Switzerland. The numbers correspond to populations listed in Appendix A. Open symbols stand for sunny ponds, filled symbols for shady ponds. Circles represent small populations, squares large populations. The star represents the city of Zürich.

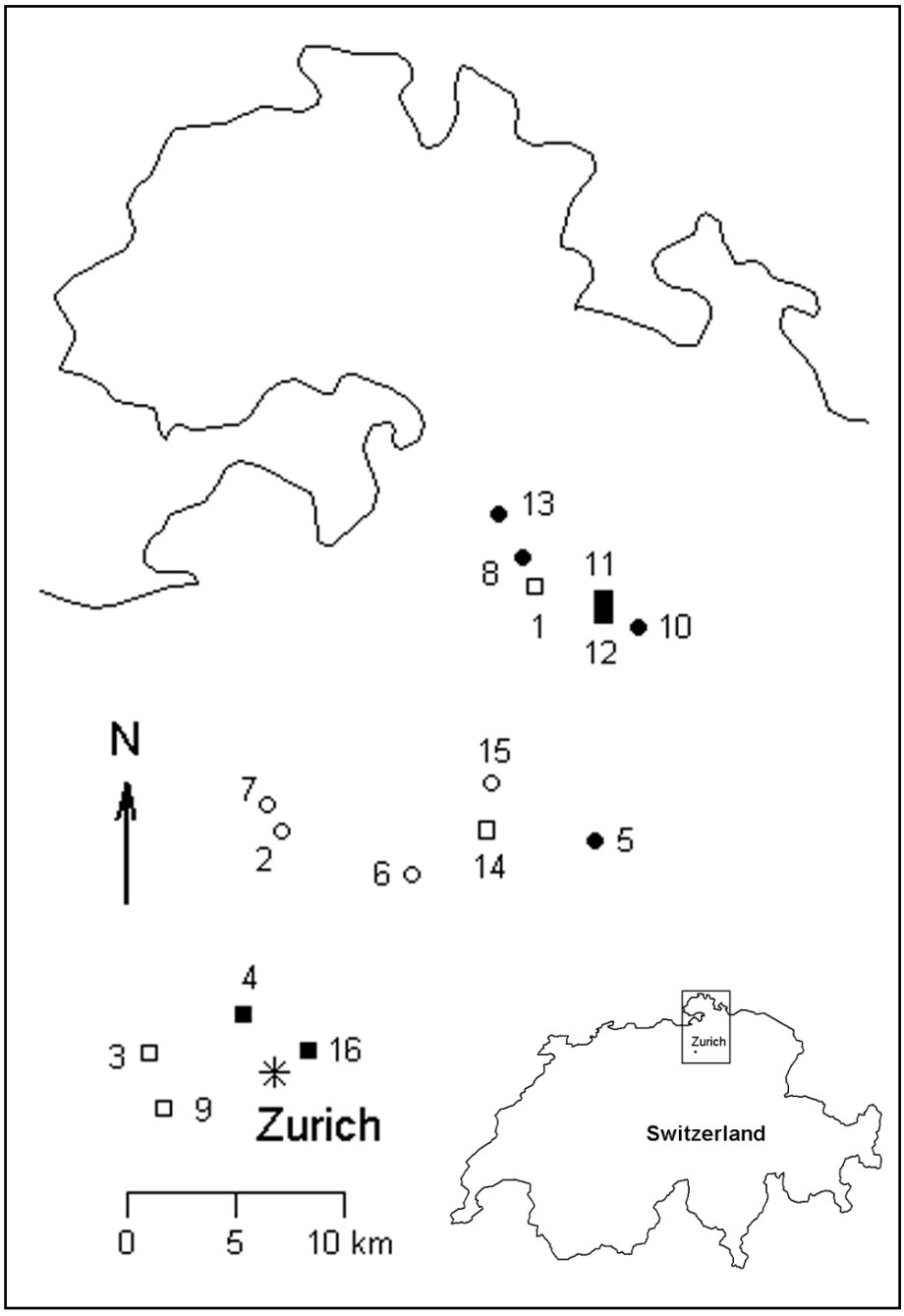


Fig. 2 – Relationship between F_{ST} values and Q_{ST} for age at metamorphosis. Each point on the figure represents a pair of ponds: open circles represent comparisons between sunny ponds, filled circles between shady, and triangles between different habitats. There was no relationship between F_{ST} values and Q_{ST}, but it is clear that shady ponds were not divergent from another (solid line), sunny ponds showed greater level of divergence (dotted line), and comparisons between different habitats showed intermediate values.

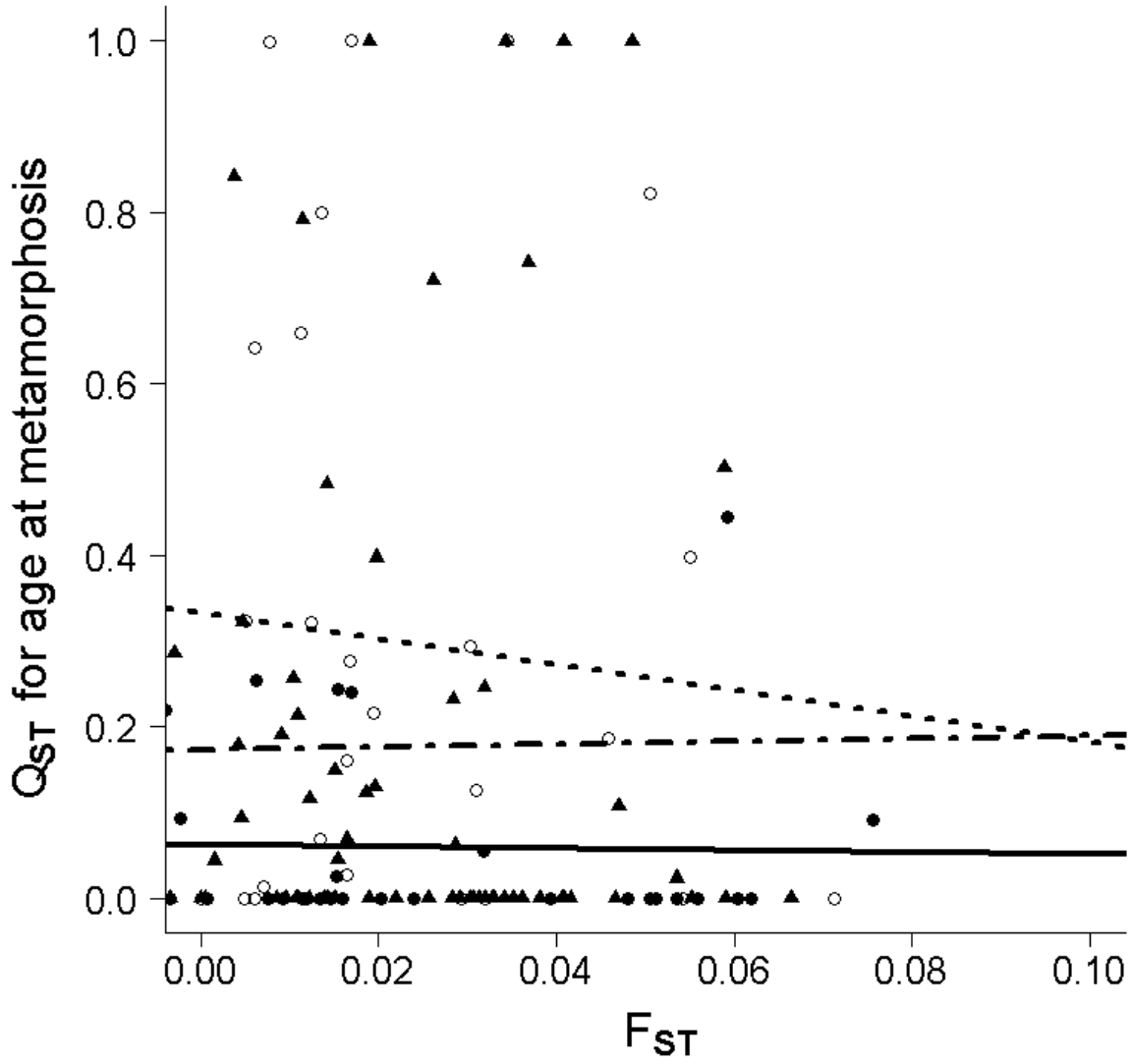
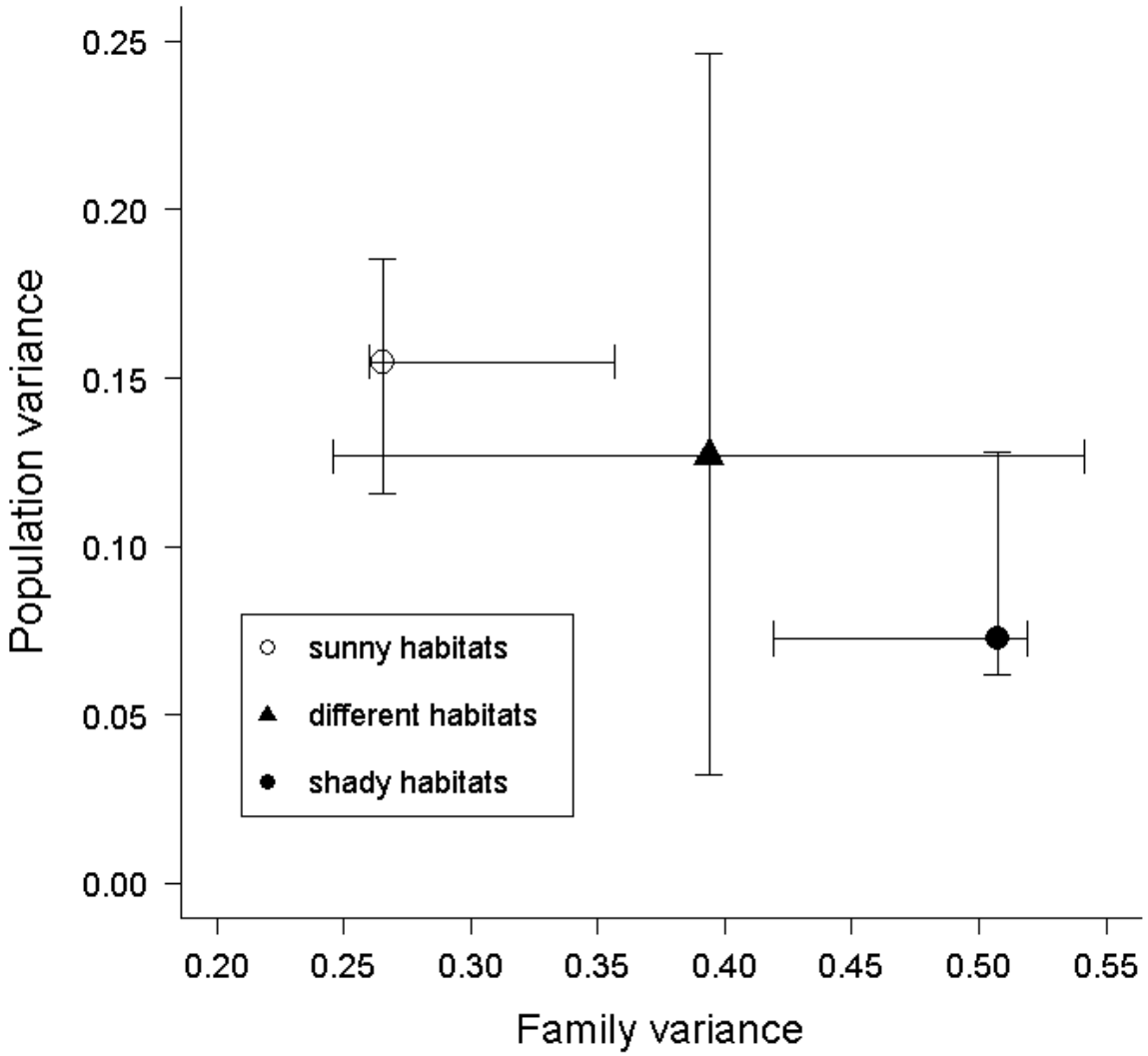


Fig. 3 – Averaged variances among families and among populations for age at metamorphosis, calculated separately according to habitat. Error bars show ± 95% confidence intervals, estimated from permutation tests. Pairs of shady ponds tended to be similar in development rate and to contain much variation among genotypes in development.



Appendices

Appendix A – Exact locations, population sizes and canopy covers of the 16 *Rana temporaria* populations.

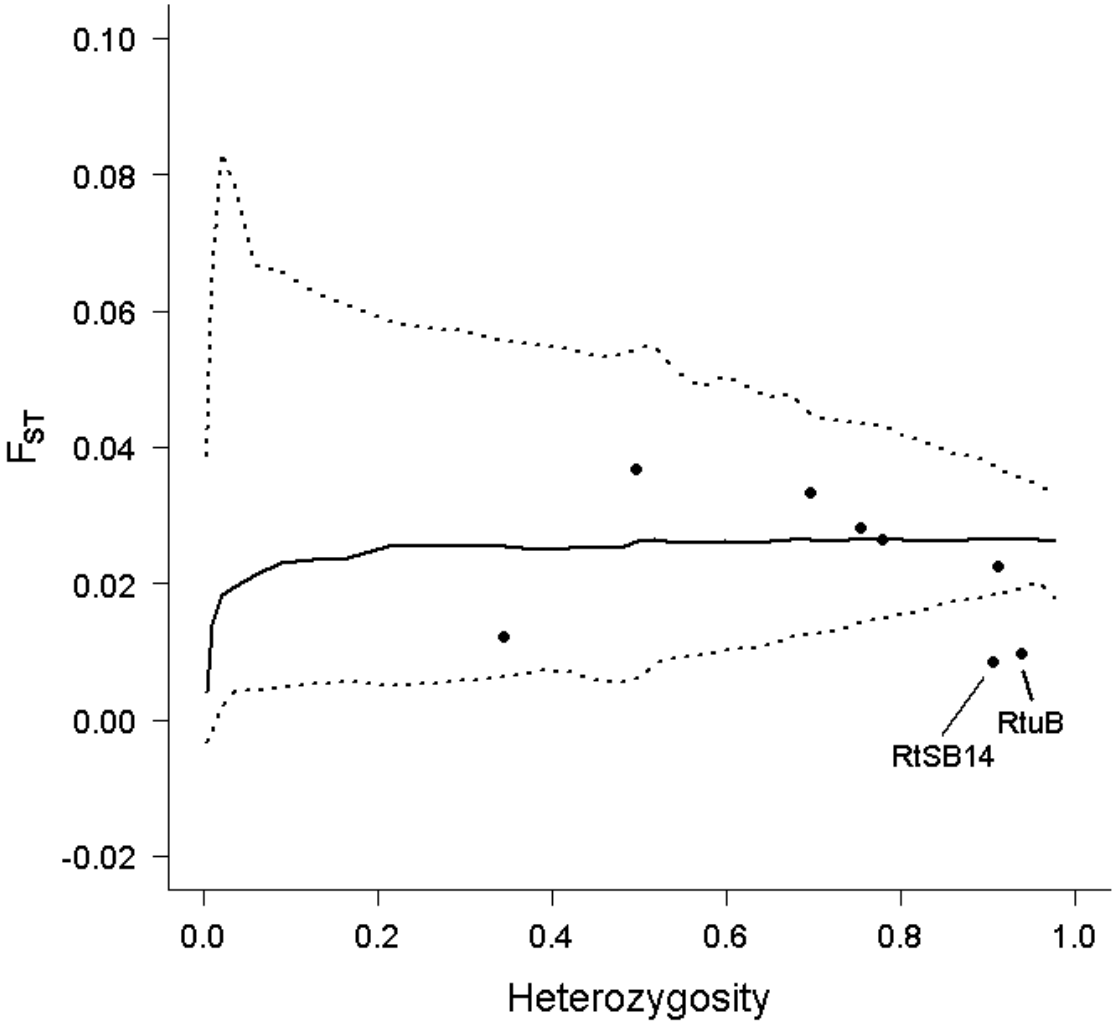
Population (Code*)	Latitude (N)	Longitude (E)	Population size†	Canopy cover‡
Adlikon (1)	47° 34' 57"	8° 41' 58"	960 (large)	0.09 (sunny)
Allmend South (2)	47° 28' 49"	8° 32' 42"	16 (small)	0.16 (sunny)
Anni's Pond (3)	47° 23' 17"	8° 27' 49"	1'091 (large)	0.03 (sunny)
Chäferberg (4)	47° 24' 13"	8° 31' 19"	605 (large)	0.52 (shady)
Eschenberg (5)	47° 28' 35''	8° 44' 13''	158 (small)	0.39 (shady)
Eigental (6)	47° 27' 45''	8° 37' 28''	30 (small)	0.11 (sunny)
Graben (7)	47° 29' 28"	8° 32' 10"	72 (small)	0.02 (sunny)
Hostbach (8)	47° 35' 41"	8° 41' 33"	50 (small)	0.37 (shady)
Hueb West (9)	47° 21' 55''	8° 28' 21"	720 (large)	0.24 (sunny)
Längeren (10)	47° 33' 56"	8° 45' 44"	94 (small)	0.64 (shady)
Oberloo East (11)	47° 34' 37''	8° 44' 32''	628 (large)	0.58 (shady)
Opfiker (12)	47° 34' 11"	8° 44' 31"	582 (large)	0.64 (shady)
Räubrichseen (13)	47° 36' 47''	8° 40' 37''	38 (small)	0.42 (shady)
Strubikon (14)	47° 28' 51"	8° 40' 10"	1'241 (large)	0.00 (sunny)
Weiertal (15)	47° 30' 00''	8° 40' 21''	65 (small)	0.09 (sunny)
Zürichberg (16)	47° 23' 21''	8° 33' 41''	808 (large)	0.33 (shady)

* population codes correspond to the numbers on Fig.1

† population size: the harmonic mean of egg clutch counts taken during 2-6 years between 1997 and 2008

‡ canopy cover: estimated by means of hemispherical photographs taken on 17-18 April and 9-10 June 2008. Depending on the size of the pond I took 3-5 photographs per pond. The normal procedure was to take a picture 2 meters from the shore on the four cardinal points and one in the middle of the pond. In very small pond I took either only 3 pictures (for small and narrow ponds: in a transect passing through the middle of the pond, two of them 2 meters from the shore, one in the middle) or only 4 pictures (for small but round ponds: on the shore on the four cardinal points). The camera was fixed on a tripod, directed toward the south, and tilted upwards at an angle of 30° above the horizon

Appendix B – Expected distribution of F_{ST} values as a function of heterozygosity, with median F_{ST} (solid line) and upper and lower 95% confidence interval (dotted lines); filled circles are the observed values for the eight microsatellite loci. Simulations were based on an Island Model (Wright, 1951) with 100 demes, 16 demes sampled, and an average of 39 individuals from each deme, infinite alleles mutational model, 20'000 realizations, using FDIST2 (Beaumont and Nichols, 1996). The results for RtSB14 and RtμB suggest homogenizing selection because populations are less differentiated than expected at these loci. The expected distribution is plotted with F_{ST} = 0.027135, as calculated after removal of these two loci.

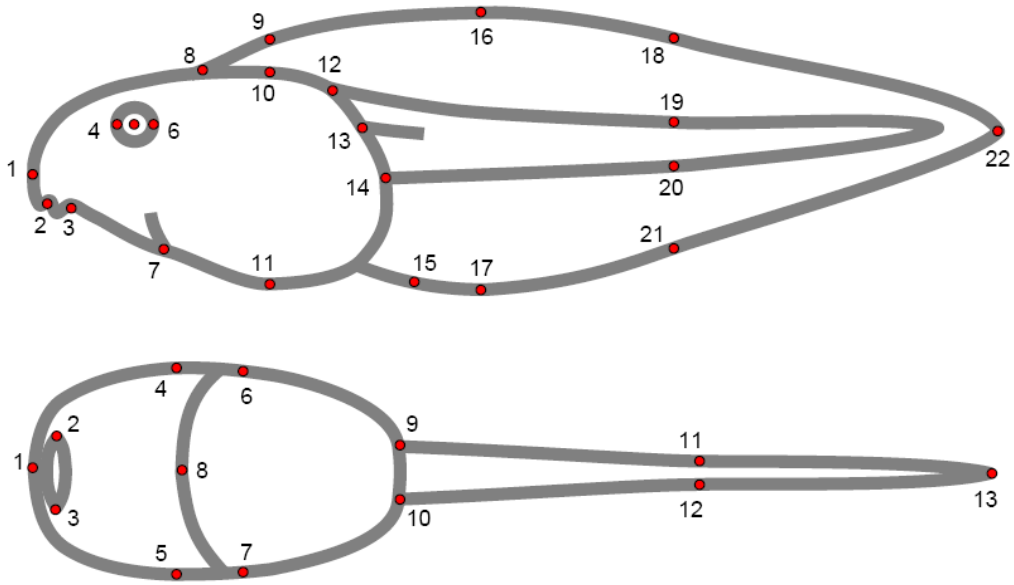


Appendix C – Genetic variation at 5 microsatellite loci of the 16 Rana temporaria populations, showing the number of individuals scored (N), mean number of alleles per locus (A), allelic richness (R_S), gene diversity (H_S), mean observed and expected heterozygosity (H_o and H_e), and inbreeding coefficient (F_{IS}). Bold FIS-values indicate populations which deviate from HWE. Standard deviations (± SD) are given for A, R_s, H_s, H_o, and H_e.

Population (Code*)	N	A (± SD)	R_S (± SD)	H_S (± SD)	H_o (± SD)	H_e (± SD)	F_{IS}
Adlikon (1)	40	7.2 ± 4.665	4.925 ± 2.710	0.643 ± 0.211	0.525 ± 0.219	0.641 ± 0.211	**0.183**
Allmend South (2)	10	4.4 ± 2.871	4.295 ± 2.717	0.564 ± 0.239	0.569 ± 0.236	0.564 ± 0.238	-0.008
Anni's pond (3)	35	6.4 ± 4.758	4.405 ± 2.558	0.617 ± 0.227	0.657 ± 0.251	0.617 ± 0.227	-0.066
Chäferberg (4)	34	5.8 ± 3.709	4.327 ± 2.084	0.630 ± 0.134	0.556 ± 0.127	0.629 ± 0.134	**0.119**
Eschenberg (5)	40	6.8 ± 4.261	4.724 ± 2.455	0.627 ± 0.218	0.545 ± 0.215	0.626 ± 0.218	**0.131**
Eigental (6)	40	6.8 ± 4.956	4.753 ± 2.588	0.626 ± 0.174	0.645 ± 0.167	0.627 ± 0.174	0.029
Graben (7)	40	7.4 ± 6.468	4.536 ± 3.053	0.573 ± 0.223	0.550 ± 0.213	0.572 ± 0.223	0.039
Hostbach (8)	39	6.6 ± 4.673	4.895 ± 2.861	0.643 ± 0.173	0.600 ± 0.194	0.643 ± 0.173	0.067
Hueb West (9)	40	6.4 ± 4.758	4.445 ± 2.560	0.639 ± 0.173	0.582 ± 0.188	0.639 ± 0.173	0.090
Längeren (10)	40	7.2 ± 4.445	5.006 ± 2.582	0.673 ± 0.175	0.602 ± 0.211	0.672 ± 0.175	**0.105**
Oberloo East (11)	42	7.2 ± 4.792	4.825 ± 2.630	0.630 ± 0.222	0.599 ± 0.215	0.629 ± 0.222	0.049
Opfiker (12)	37	7.6 ± 5.748	5.131 ± 3.036	0.628 ± 0.229	0.551 ± 0.227	0.627 ± 0.229	**0.122**
Räubrichseen (13)	34	7.6 ± 5.083	4.977 ± 2.766	0.605 ± 0.246	0.550 ± 0.248	0.604 ± 0.246	0.091
Strubikon (14)	37	7.6 ± 5.238	4.720 ± 2.572	0.604 ± 0.188	0.562 ± 0.197	0.604 ± 0.188	0.070
Weiertal (15)	21	6.2 ± 3.429	5.074 ± 2.588	0.680 ± 0.185	0.571 ± 0.141	0.677 ± 0.183	**0.160**
Zürichberg (16)	40	6.0 ± 3.847	4.373 ± 2.102	0.595 ± 0.205	0.550 ± 0.177	0.595 ± 0.205	0.076

* population codes correspond to the numbers on Fig.1

Appendix D – Landmarks used for the morphology analysis (Van Buskirk)



Side-view landmarks

1 Most anterior point on the nose
2 Center of the partially-opened mouth when viewed from the side
3 Junction of the posterior edge of the lower labium and the body wall
4 Anterior edge of the iris on a horizontal line extending through the center of the eye
5 Center of the pupil
6 Posterior edge of the iris on a horizontal line extending through the center of the eye
7 Lower edge of the head/body at the anterior gut margin
8 Point at which the edge of the dorsal fin attaches to the top of the head/body
9 Highest point of the head/body or tail fin at 2/3rds of the distance between # 1 and # 14
10 Dorsal edge of the head/body at 2/3rds of the distance between # 1 and # 14
11 Ventral edge of the head/body at 2/3rds of the distance between # 1 and # 14
12 Point where the upper edge of the tail muscle meets the head/body
13 Point where the notochord (identified from the pattern of myotomes) meets the head/body
14 Point where the bottom edge of the tail muscle meets the head/body
15 Point where the center of the anus meets the lower edge of the tail fin
16 Dorsal edge of the tail fin at the deepest point
17 Ventral edge of the tail fin directly below # 16
18 Upper edge of the tail fin halfway between # 14 and # 22
19 Top of the tail muscle halfway between # 14 and # 22
20 Bottom of the tail muscle halfway between # 14 and # 22
21 Ventral edge of the tail fin halfway between # 14 and # 22
22 Tip of the tail fin

Bottom-view landmarks

1 Most anterior point of the nose
2 Left edge of the mouth, where the anterior and posterior labial tooth rows converge
3 Right edge of the mouth, where the anterior and posterior labial tooth rows converge
4 Left edge of the body at the widest point anterior to spiracle
5 Right edge of the body at the widest point anterior to spiracle
6 Left edge of the body where the intestinal mass is the widest
7 Right edge of the body at the widest part of the gut mass
8 Separation of the head and the gut at the midline
9 Point where the left edge of the tail muscle intersects the body
10 Point where the right edge of the tail muscle intersects the body
11 Left tail muscle edge halfway between # 9/10 and # 13
12 Right tail muscle edge halfway between # 9/10 and # 13
13 Tip of the tail fin

Appendix E – Pairwise F_{ST} comparisons for the 16 *Rana temporaria* populations. Significant population differentiation is denoted with bold values (P < 0.05: P-values are obtained after 12'000 randomizations by permuting genotypes among samples after standard Bonferroni correcture (adjusted nominal level 5% for multiple comparisons) α' = 0.05 / 120 = 0.000417.

Population	Adli	Allm	Anni	Chaf	Esch	Eige	Grab	Host	Hueb	Lang	Ober	Opfi	Raub	Stru	Weie	Zuri
Adlikon	-															
Allmend South	0.017	-														
Anni's pond	0.020	**0.054**	-													
Chäferberg	**0.041**	**0.049**	**0.032**	-												
Eschenberg	0.014	**0.055**	**0.019**	**0.048**	-											
Eigental	0.011	0.034	0.020	0.033	0.004	-										
Graben	0.006	0.013	**0.046**	**0.059**	**0.028**	0.012	-									
Hostbach	0.012	0.042	**0.047**	**0.062**	0.015	0.002	**0.015**	-								
Hueb West	**0.016**	**0.051**	0.005	0.026	**0.009**	0.014	**0.039**	**0.032**	-							
Längeren	0.000	**0.031**	**0.033**	**0.032**	**0.016**	0.010	**0.015**	0.008	**0.019**	-						
Oberloo East	-0.003	0.038	**0.036**	**0.059**	**0.016**	0.017	0.010	0.015	**0.031**	0.000	-					
Opfiker	0.004	0.034	**0.035**	**0.060**	**0.017**	0.011	0.005	0.012	**0.037**	0.006	-0.002	-				
Räubrichseen	0.009	0.059	**0.047**	**0.076**	**0.013**	0.020	0.014	0.020	**0.041**	0.012	-0.004	-0.004	-			
Strubikon	0.030	**0.071**	**0.032**	**0.055**	0.000	0.000	0.031	0.014	0.017	**0.030**	**0.034**	**0.028**	**0.029**	-		
Weiertal	0.008	0.055	0.017	0.019	-0.003	0.006	0.029	0.015	0.005	0.005	0.012	0.012	0.011	0.007	-	
Zürichberg	**0.041**	**0.066**	0.022	0.009	**0.024**	0.020	**0.054**	**0.054**	**0.026**	**0.039**	**0.051**	**0.051**	**0.056**	0.029	0.011	-

Appendix F – Significance of variation among families and among populations for each trait, based on log-likelihood tests from mixed-effects models (in bold significant values).

	Among families		Among populations	
Trait	LR-statistic	*P*-value	LR-statistic	*P*-value
Life-history traits				
Initial mass	0.000	1	12.481	**0.0004**
Mass at day 36	16.987	**<.0001**	6.602	**0.0102**
Early growth rate (days 0-36)	18.934	**<.0001**	7.721	**0.0055**
Mass at metamorphosis	9.423	**0.0021**	2.366	0.1240
Age at metamorphosis	6.281	**0.0122**	2.539	0.1111
Survival	6.166	**0.0130**	0.136	0.7120
Morphometric traits (expressed as shapes)				
Bottom RW1	0.000	0.9997	0.422	0.5160
Bottom RW2	7.186	**0.0073**	8.826	**0.0030**
Side RW1	4.389	**0.0362**	1.935	0.1642
Side RW2	4.004	**0.0454**	5.562	**0.0183**
Side RW3	0.271	0.6024	0.000	0.9997
Side RW4	5.177	**0.0229**	1.003	0.3165
Side RW5	5.781	**0.0162**	2.216	0.1366
Morphology traits (mm)				
Head length	4.579	**0.0324**	3.393	0.0655
Head depth	3.997	**0.0456**	3.290	0.0697
Head width	2.047	0.1525	1.522	0.2173
Mouth width	0.000	0.9996	0.000	0.9995
Tail length	8.578	**0.0034**	9.984	**0.0016**
Maximum tail depth	23.549	**<.0001**	1.129	0.2881
Tail depth at ½	11.280	**0.0008**	0.123	0.7262
Tail muscle depth at ½	19.871	**<.0001**	1.681	0.1947
Tail muscle width at the base	0.641	0.4232	0.000	0.9994
Behaviour				
Active tadpoles	10.619	**0.0011**	6.163	**0.0130**
Hiding tadpoles	2.792	0.0947	12.618	**0.0001**

CONTRIBUTION OF GENE FLOW TO EFFECTIVE POPULATION SIZES

Abstract

In conservation biology, the estimation of effective population size N_e is a central concern, because it is strictly related to the amount of genetic variation present within a population, and thus to its evolutionary potential. However, using the census size of a population, N_c, to correctly estimate N_e is notorious difficult, as many factors may impact it. In this context, the spatial genetic structure of the populations under consideration is also important. Here, I took genetic data as reflection of N_e for a set of frog populations. By combining landscape information with census size data, I estimated the contribution of surrounding breeding sites to the observed genetic variation in the target populations and identified relevant factors impacting gene flow. Genetic variation was not explained only by the target population, but instead required additional contribution from other breeding sites in the surroundings, suggesting a prominent role of gene flow. Moreover, dispersal decreased with distance, with roads impacting it negatively, whereas there was some evidence that forest may favour dispersal. Thus, care has to be taken when identifying population borders for conservation or management, also by considering the surrounding landscape. I have also shown that inference from genetic data might work better for allelic richness than observed heterozygosity, because the latter can be impacted by past demographic histories.

Introduction

Genetically effective population size, N_e, considers only those individuals that successfully contribute gametes to the next generation. The estimation of N_e is a central concern in conservation biology, because genetic variation, the raw material for evolutionary changes, is strictly related to population size. In fact, the magnitude of drift, which decreases genetic variation, is inversely proportional to N_e, i.e. $1/2N_e$ per generation (Kimura, 1955). A positive relationship between N_e and genetic variation (allelic diversity and heterozygosity) at neutral loci at equilibrium is predicted by neutral theory (Kimura, 1983) and has been confirmed by empirical studies (Soulé, 1976; Frankham, 1996; Spielman *et al.*, 2004).

The effective population size is defined as the size of an idealized population that would experience the same amount of drift or inbreeding as the population under consideration (Wright, 1931; 1938). As it relies on assumptions that are fairly unrealistic in natural settings, N_e can be very different from the census size of a population, N_c, and is usually much less. Frankham (1995) found that N_e averaged only 10% of N_c in a survey of published estimates. Moreover, the ratio between N_e and N_c is highly variable because N_e is influenced by many factors that differ among species (Frankham, 1995). These include fluctuations in population size, unequal sex ratio, variation in breeding success and overlapping generations (Wright, 1931; 1938; Crow and Kimura, 1970). Various methods have been suggested to estimate N_e, either directly from demographic data or indirectly from genetic markers. However, estimation of N_e still remains problematic. There is no general formula that accounts for demography and all the other factors known to be important, as reviewed by Caballero (1994). Indirect estimates of N_e from genetic data also have limitations (e.g. Beaumont, 2003).

One factor that tends to decrease N_e is spatial genetic structure. Therefore, it is important to correctly define which unit should be considered as a population. This problem is easily overlooked in pond-breeding amphibians, because breeding sites are spatially discrete and the animals are relatively philopatric (e.g., dispersal rates between breeding sites are low). It is therefore natural that most workers consider breeding sites to be populations. However, if dispersal occurs between breeding sites, this will cause N_e to be greater than expected based on the number of animals at the spot. Some studies of N_e in amphibians have suggested that gene flow among subpopulations is high. For example, Schmeller & Merilä (2007) found great differences between demographic and genetic estimates of N_e in *Rana temporaria* (Linnaeus), and true N_e was much higher than N_c because of gene flow. Indirect evidence came also from the study of Brede and Beebee (2004), who compared genetic variation and N_c in two sympatric anuran species with similar autecologies. *Rana temporaria* displayed higher levels of genetic variation than the common toad *Bufo bufo*, despite its smaller census size, suggesting a prominent role of gene flow for the maintenance of high levels of genetic diversity.

Thus, accurate estimation of N_e depends on accounting for a rather complex list of factors that influence it. These factors may be species specific, such as sex ratio, degree of population fluctuation, or propensity to disperse. They may also relate to the local habitat or the surrounding landscape, if that in turn influences gene flow. The impact of gene flow on the object of my study, *R temporaria*, is not known. In this study I aimed to determine the exact population structure of *R. temporaria* populations occurring at small spatial scale, thus the importance of gene flow in this system. By integrating demographic data, which accounted for fluctuating population sizes, and neutral genetic variation, which I assumed to be an accurate reflection of the true N_e, I inferred spatial structure. Combining these with elements from a

landscape genetics approach (Manel *et al.*, 2003) allowed me to identify dispersal distances and the factors that may promote or hinder gene flow.

Material & Methods

Study species and populations

Rana temporaria is a widespread amphibian in Europe, with a very broad habitat range with respect to altitude (from sea level to more than 2'000 m in the Alps) and latitude (from Italy to north of the Arctic Circle). *Rana temporaria* is a habitat generalist during the larval stage as well, occurring in every water-body from small puddles to large lakes (Nöllert and Nöllert, 1992). This makes *R. temporaria* a good potential migrant compared to other more specialized species; dispersal distances are usually less than 2 km (Seitz *et al.*, 1992; Rittenhouse and Semlitsch, 2007; Semlitsch, 2008).

I sampled 16 populations in 2008 in northeastern Switzerland within a total area of approximately 750 km^2 (Fig. 1; exact locations and details are given in Appendix A). Population size, term used here to refer to N_c, corresponds to the number of breeding females, which was estimated from clutch counts during March 1997-2009. *Rana temporaria* is known to have fluctuating population sizes (Meyer *et al.*, 1998; Alford and Richards, 1999), so I calculated the harmonic mean of clutch counts to obtain a long-term average appropriate for fluctuating population sizes (Frankham *et al.*, 2004, p. 63). Harmonic mean clutch counts ranged from 11 to 1'872 (mean = 556).

For these 16 populations, hereafter called target populations, I checked all surrounding area within a 2-km radius for other *R. temporaria* clutches. This was done during at least one year and up to 9 years during March 1997-2009 (average of

5 years for each target population, 3 years for all breeding sites). The surrounding non-target breeding sites are hereafter called peripheral populations.

Genetic analyses

In March 2008, I collected an average of 35.6 eggs per target population for estimating genetic variation. This gave a total of 569 eggs (range 10-42 per population). After hatching, larvae were preserved at -20°C in alcohol until DNA extraction. To estimate genetic variation, I scored each specimen at 8 microsatellite loci: Rtempμ4, Rtempμ7 (Rowe and Beebee, 2001), RtμR, RtμB, RtμP (Pidancier *et al.*, 2002), Rt2Ca2-22, Rt2Ca30 (Teacher *et al.*, 2009) and RtSB14 (Berlin *et al.*, 2000). As in previously described protocols (Chapter 2), I extracted the DNA and processed the PCR products with an ABI 3730 sequencer. Alleles were scored with GeneMapper Software (version 3.7, Applied Biosystem).

The microsatellite data were first screened for null alleles using MICRO-CHECKER version 2.2.3 (VanOosterhout *et al.*, 2004; available at http://www.microchecker.hull.ac.uk). Where null allele presence was detected, I adjusted genotype frequencies based on Brookfield Estimator 2 (Brookfield, 1996), under the assumption that a single null allele was present. Selective neutrality was tested with FDIST2 (Beaumont and Nichols, 1996; available at www.rubic.rdg.ac.uk/~mab/software.html). In the end, the loci RtSB14 and RtμB were discarded from analysis because of evidence of homogenizing selection. I also excluded the locus Rt2Ca30 because it had a null allele frequency of 20-40% in all 16 target populations. This left 5 microsatellite loci for estimating genetic variation.

Allelic richness (R_S, El Mousadik & Petit, 1996) was assessed with FSTAT 2.9.3.2 (Goudet, 1995), and observed and expected heterozygosity (H_o and H_e) with ARLEQUIN version 3.1 (Excoffier *et al.*, 2005).

Landscape analysis

I first evaluated landscape features within 2 km of each target population using Swiss topographical maps at a scale of 1:25'000 (Andelfingen, ed. 2004; Bülach, ed. 2003; Winterthur, ed. 2004; Zürich, ed. 2003). For every peripheral population, I recorded the land use and barrier types encountered along a straight-line path connecting it to the target population. Straight-line dispersal paths were assumed because ranid frogs seem to disperse in straight lines (Goldberg and Waits, 2010, and references therein). Land use fell under five categories (urban, roads, open field, marsh, and forest). Urban areas were defined as aggregates of buildings occupying more than 1 ha. Barriers were accounted for in two ways. In analyses of land use, the category termed roads was measured as the distance covered by concrete or rails that a dispersing frog would cross. In analyses of barrier elements, obstacles to dispersal were counted within two categories: medium barriers (defined as 1st and 2nd class roads after Swiss topographical map classification) and large barriers (highways, airport runways, and railways).

The analysis was designed to express genetic variation, which I assume to be proportional to N_e, as some function of target population size and the sizes of all peripheral populations. I built a total of 21 generalized linear models, each assuming a Gaussian distribution and identity link function, and used a multi-model inference approach to evaluate the relative support in the data for each model (Burnham and Anderson, 2002). This enabled me to determine the most important predictors of effective population sizes. Appendix B lists all models. Every model included target population size, because I assumed that individuals breeding locally always contribute to N_e. Some models might have been excluded *a priori* but were tested for logical completeness. Population sizes (plus 1) were log-transformed because the

logarithm of population size is the best predictor of neutral variation (Frankham, 1996).

The simplest model included only target population size. This model assumes that dispersal from nearby breeding sites has no influence on effective population size. All other models included the sizes of peripheral populations, assuming that all breeding individuals within a 2-km radius combine in some way to influence N_e. Peripheral populations were also expressed as relative sizes, defined as ratio between peripheral size and target size. Here the idea was that neighboring populations affect target populations in proportion to their relative sizes. The first of these models assumed that all peripheral populations contribute equally, which would occur if dispersal is not affected by distance. The other models weighted peripheral population sizes by their distances from the target population and by the number of barriers that would be crossed by dispersing frogs. Barrier weights were expressed as [population size / (2 * *k* * number of barriers)]; k = 1 for medium barriers and k = 2 for large barriers, assuming that large barriers have twice as large an effect of gene flow as medium barriers. Geographic distances were weighted according to three functions that all assume that dispersal among ponds decreases with distance: linear (-0.5*distance), negative exponential ($e^{-1*distance}$), and sigmoid ($20 / [19+e^{4*distance}]$) (see Appendix C). Some models accounted for land use along the dispersal path in order to detect factors that favour or hinder dispersal. In the models with land use, I first ran a model including all possible land uses, and then retained in the final models to be compared only those factors which had a p-value of < 0.3. This was done in order to avoid overparameterization and the risk of missing the relevant land uses that affect dispersal.

Before constructing models, I checked for correlations among the independent variables. Land uses were not strongly correlated (all p-values > 0.538), but barriers

were strongly associated with distances (all p-values < 0.007). Therefore, I did not include models with both barriers and land uses together.

Statistical analyses were conducted in R 2.14.0 (R Development Core Team, 2010). Model selection was done with the package "AICcmodavg" (Mazerolle, 2011), which ranks the models according to their AIC_c (Akaike Information Criterion, corrected for small sample size) and also yields delta AIC_c and Akaike weights. Parameter estimation and confidence intervals were calculated with the package "MuMIn" (Barton, 2011).

Results

Genetic analyses

Across loci, the number of alleles sampled ranged from 3 to 29 (average 12.2), and allelic richness (R_S) ranged from 2.15 to 9.78 (average 5.07). Across populations, R_S ranged from 4.295 to 5.302 (average 4.815), observed heterozygosity (H_o) from 0.550 to 0.657 (average 0.603), and expected heterozygosity (H_e) from 0.564 to 0.692 (average 0.631). Appendix D reports values for each population separately. H_o and R_s were not correlated (p-value = 0.247).

Landscape analysis

Observed heterozygosity, H_o, was best explained by two models that included the sizes of peripheral populations weighted by distance through forested land and roads, weighted either sigmoidally or linearly (Table 1A). Values of R^2 were above 60% for both models. Unexpectedly, increasing numbers of peripheral clutches had negative impacts on H_o, with the effect of roads about 2-3 times more negative than that of forest (Table 2A).

For allelic richness, R_s, six models fell within 2 AIC_c units of the best model, although no model had an R^2 over 42.2% (Table 1B). The important variables here were the size of peripheral populations weighted by either 1 or by an exponentially or linearly decreasing function of distance. Distances through roads and forests were again important. Peripheral clutches separated by forest had a slightly significant positive impact on R_s, whereas roads slightly negative (Table 2B). A few of the best models also included peripheral population sizes weighed by large barriers and by peripheral sizes relative to the target size.

The predicted genetic variation from the best models was closely related to the observed values for both H_o ($R^2 = 0.674$, $p < 0.0001$) and R_s ($R^2 = 0.499$, $p = 0.002$) (Figs. 2a and 2b). Predicted values of both measures were expressed as target population size and peripheral population sizes weighted by distance through forest and across roads; these distances were weighted with a sigmoidal function for H_o and a linear function for R_s. Figures 2c and 2d depict the effect of target population size on H_o and R_s. In the case of R_s, the relationship was not significant. Figures 2e and 2f display the negative effect of peripheral clutches separated by forest on H_o and their positive effect – although not quite significant – on R_s. Weighing peripheral clutches with distance as linear (for H_o) or negative exponential functions (for R_s) of distance generated similar results.

Discussion

This study began with genetic data that reflect effective population size (N_e) for a set of frog populations, and estimated the contribution of surrounding breeding sites to the maintenance of genetic variation in the target populations. My approach combined landscape information with demographic data to reveal factors that promote or hinder gene flow. The main finding was that genetic variation is not

explained by the number of females ovipositing in the target breeding locality, but instead requires additional information about the number of females occupying the surrounding landscape. This suggests a prominent role of gene flow in maintaining neutral genetic variation in these frog populations. Moreover, dispersal decreased with distance, with roads impacting it negatively, whereas there was some evidence that forest may favour dispersal.

The two measures of neutral genetic variation used here – observed heterozygosity H_o and allelic richness R_s – were not correlated with each other. More surprising, as the numbers of clutches in the landscape increased, H_o decreased, as evidenced by the negative and significant model-averaged coefficients in Table 2A. It is possible that H_o is not a good measure for these populations, because it reflects past history more than recent dynamics. In fact, the predicted positive relationship between H_o and N_e depends on populations being at mutation-drift equilibrium, and this may require a very long time (Whitlock and McCauley, 1999). I can infer that populations of most amphibians are not at equilibrium because they fluctuate considerably (Alford and Richards, 1999), indicating that the importance of drift varies among years (Whitlock, 1992). In this case, populations with identical values of N_e at a particular point in time may have different H_o because of their different histories and different initial heterozygosities (Podolsky, 2001). In spite of these considerations, it was nevertheless amazing to find such a strong and significant negative relationship between H_o and the number of clutches in the surrounding landscape. Most other published studies report that H_o increases with N_c (Frankham, 1996), even though these populations are also unlikely to be at mutation-drift equilibrium.

I propose the following explanation based on the differential impact of gene flow on large and small populations. Imagine that bottlenecks at breeding sites happen regularly because of the dynamic nature of the landscape and the small number of

frog that colonize new ponds. Bottlenecks cause genetic erosion, especially measured with R_s (Nei *et al.*, 1975). These bottlenecks tend to be of short duration; populations occupying new habitats soon grow at a size determined by the suitability of the local larval and adult habitat, and they begin to exchange migrants. At this stage, those populations that are small, and are surrounded by few other breeding sites, are more strongly affected by immigration than are large populations. In particular, heterozygosity in small populations increases very quickly because newly-arriving alleles are immediately at relatively high frequency. The same number of immigrants arriving in a large population also bring novel alleles with them, but at such low frequency that they have little effect on H_o. The result of this process is that high heterozygosity occurs in the smallest populations with the fewest nearby sites. There are in fact other examples in which diverse population histories have eliminated the correlation between population size and H_o (e.g. Ellstrand and Elam, 1993; Podolsky, 2001; Willi and Määttänen, 2011). Nevertheless, I still think that genetic variation reflects long-term effective population size. Thus, depending on the factors influencing the dynamics of your populations, you better choose R_s than H_o, which will more accurately reflect the current levels of variation. Finally, R_s seems more important than H_o for long-term responses of populations (Allendorf, 1986), and might therefore better predict N_e.

Gene flow among *Rana temporaria* breeding sites is evidently common, and responsible for the maintenance of genetic variation within populations. This finding is supported by other studies of the same species. For example, Schmeller & Merilä (2007) compared direct and indirect estimates of N_e to detect the factors that are influencing N_e. Their discovered that N_e was badly underestimated by N_c, suggesting a prominent role of gene flow in the two populations under study. Brede & Beebee (2004) found higher levels of genetic variation in spite of lower N_c in *R. temporaria*

compared to the common toad *Bufo bufo*. The two species are sympatric and share many relevant biological and habitat features, but the authors suggested that a higher migration rate in *R. temporaria* could explain its higher H_o. However, the contribution of dispersal depends not only on the species, but also on the surrounding landscape. It has been shown that frog populations occurring in urban areas were far more differentiated (over only 2.3 km) than populations in rural areas over an average of 40 km (Hitchings and Beebee, 1997). Here, I too observed that dispersal decreases with distance. There was no clear evidence supporting any single dispersal kernel over the others (linear, exponential or sigmoidal). Mark-recapture studies on amphibians and many other organisms suggest that dispersal kernels are usually best expressed as a negative exponential decrease function (e.g. Berven and Grudzien, 1990; Trenham *et al.*, 2001). The wood frog *Rana sylvatica* is pretty similar to the common frog, and only juveniles dispersed on average about 1.2 km. As reviewed by Smith & Green (2005), the absolute farthest dispersal distance can be about 12 km for anurans, although only very few individuals may be able to migrate that far and most amphibians do not disperse that much. However, dispersal in amphibians is still a quite debated field, as precise tracking of individuals is difficult and time consuming. Moreover, differences between juveniles and adults (Berven and Grudzien, 1990), as well as between sexes exist (Semlitsch, 2008), thus making general conclusions hard to infer. As hypothesized, roads affected negatively amphibian migration, whereas forest had a slightly positive effect. This confirms the findings of previous studies, in which forest favoured gene flow among amphibian populations (Emaresi *et al.*, 2011), whereas roads had a negative impact (reviewed by Holderegger and Di Giulio, 2010).

Little is known about the relationship between population size and dispersal, although there are many models depicting how genetic variation is related to N_e

under dispersal (reviewed in Felsenstein, 1976). As discussed by Willi *et al.* (2006), in some cases, immigration and emigration rates are positively related to local population size. At the same time, immigrants into small populations might be favoured by selection. Here, I tried to calculate gene flow effects according to the population sizes with the so-called relative population sizes, expressed as ratio between peripheral size and target size. For R_s, this variable appeared to be important to some extent, and at least considering factors that promote dispersal, as suggested by the positive effect of relative sizes weighted by distance through forest.

The approach developed here recognized that the effective population size sampled at a particular site arises from the efforts of individuals that breed locally combined with those that breed nearby and (occasionally) contribute gene flow to the local site. This approach addresses the widespread appreciation that genetic populations extend well beyond the collection of individuals observed to interact regularly. There was some indication that high levels of dispersal can increase N_e. In this system, *R. temporaria* populations extend well beyond their breeding sites, highlighting that care has to be taken when identifying population borders for conservation or management. A particular effort has to be done in order to recognize the true population units, also by considering the surrounding landscape. I have also shown that inference from genetic data might work better for allelic richness than observed heterozygosity. In this context, it is important to recognize past demographic histories, as well as the ecology and the mating-system of the species, and the genetic structure of the populations, which could affect observed variation.

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Tables

Table 1 – Summary of model inference to predict genetic variation as a function of target and peripheral clutch counts. The table documents all models that fell within 2 AICc units of the best model for observed heterozygosity (A) and for allelic richness (B). For each model, Δ_i gives the difference from the best model in AICc and w_i gives the Akaike weight, which sums to 1 across all models. LL is the log-likelihood and R^2 the percentage of explained variation for each model. In (A), the second-best model is included because it fell only slightly more than 2 AIC_c units from the best model; no other model occurred within 6 AIC_c units.

A. Models for observed heterozygosity within 2 AIC units of best model

Model†	AIC_c	Δ_i	w_i	LL	R^2 (%)
tar.size + per.size * sigm * (roads + forest)	-62.36	0.00	0.74	39.18	67.4
tar.size + per.size * lin * (roads + forest)	-60.30	2.05	0.26	38.15	62.7

B. Models for allelic richness within 2 AIC units of best model

Model†	AIC_c	Δ_i	w_i	LL	R^2 (%)
tar.size + per.size * lin * (roads + forest)	14.94	0.00	0.25	0.53	42.2
tar.size + per.size * exp * (roads + forest)	15.75	0.80	0.17	0.13	42.0
tar.size + per.size	16.12	1.17	0.14	-2.24	27.6
tar.size + per.size * large barriers	16.13	1.19	0.14	-2.25	28.2
tar.size + per.size * exp	16.46	1.52	0.12	-2.41	26.6
tar.size + rel.size * exp * forest	16.78	1.84	0.10	-2.57	20.4
tar.size + per.size * lin	16.79	1.85	0.10	-2.58	25.4

† the following abbreviations are used: “tar.size” for target population size; “per.size” for peripheral population size; “rel.size” for relative peripheral population size; “sigm”, “lin”, “exp” for weighing peripheral population sizes by distance expressed as sigmoid, linear, exponential decrease function; “roads” and “forest” stand for the respective land uses. For details of the calculations see “Material & Methods”

Table 2 – **Parameter estimates from the models given in Table 1 for predicting genetic variation (A: observed heterozygosity; B: allelic richness) as a function of target and peripheral population sizes. In part A, bold text indicates 95% confidence intervals (CIs) that do not include 0. In part B, parameters were not significant, but bold text indicates those effects for which the 90% CI did not include 0.**

A. Model-averaged parameter estimates for observed heterozygosity

Parameter	Coefficient	Lower 95% CI	Upper 95% CI
(Intercept)	**0.731**	**0.652**	**0.810**
Target pop size	**-0.011**	**-0.021**	**-0.010**
Peripheral size * sigmoid * roads	**-0.032**	**-0.049**	**-0.014**
Peripheral size * sigmoid * forest	**-0.011**	**-0.019**	**-0.003**
Peripheral size * linear * roads	**-0.023**	**-0.036**	**-0.010**
Peripheral size * linear * forest	**-0.014**	**-0.023**	**-0.005**

B. Model-averaged parameter estimates for allelic richness

Parameter	Coefficient	Lower 95% CI	Upper 95% CI
(Intercept)	**4.382**	**3.028**	**5.736**
Target pop size	-0.023	-0.180	0.134
Peripheral size	**0.119**	**-0.004**	**0.242**
Peripheral size * exponential	**0.124**	**-0.010**	**0.258**
Peripheral size * linear	**0.112**	**-0.014**	**0.239**
Peripheral size * large barriers	**0.120**	**-0.004**	**0.244**
Peripheral size * linear * roads	**-0.117**	**-0.254**	**0.020**
Peripheral size * linear * forest	**0.096**	**-0.002**	**0.193**
Peripheral size * exponential * roads	-0.102	-0.237	0.033
Peripheral size * exponential * forest	**0.108**	**-0.003**	**0.219**
Relative size * exponential * forest	**0.219**	**-0.028**	**0.466**

Figures

Fig. 1 – Map showing the location of the 16 study populations in northern Switzerland. The numbers correspond to populations listed in Appendix A. The square symbol represents Zürich.

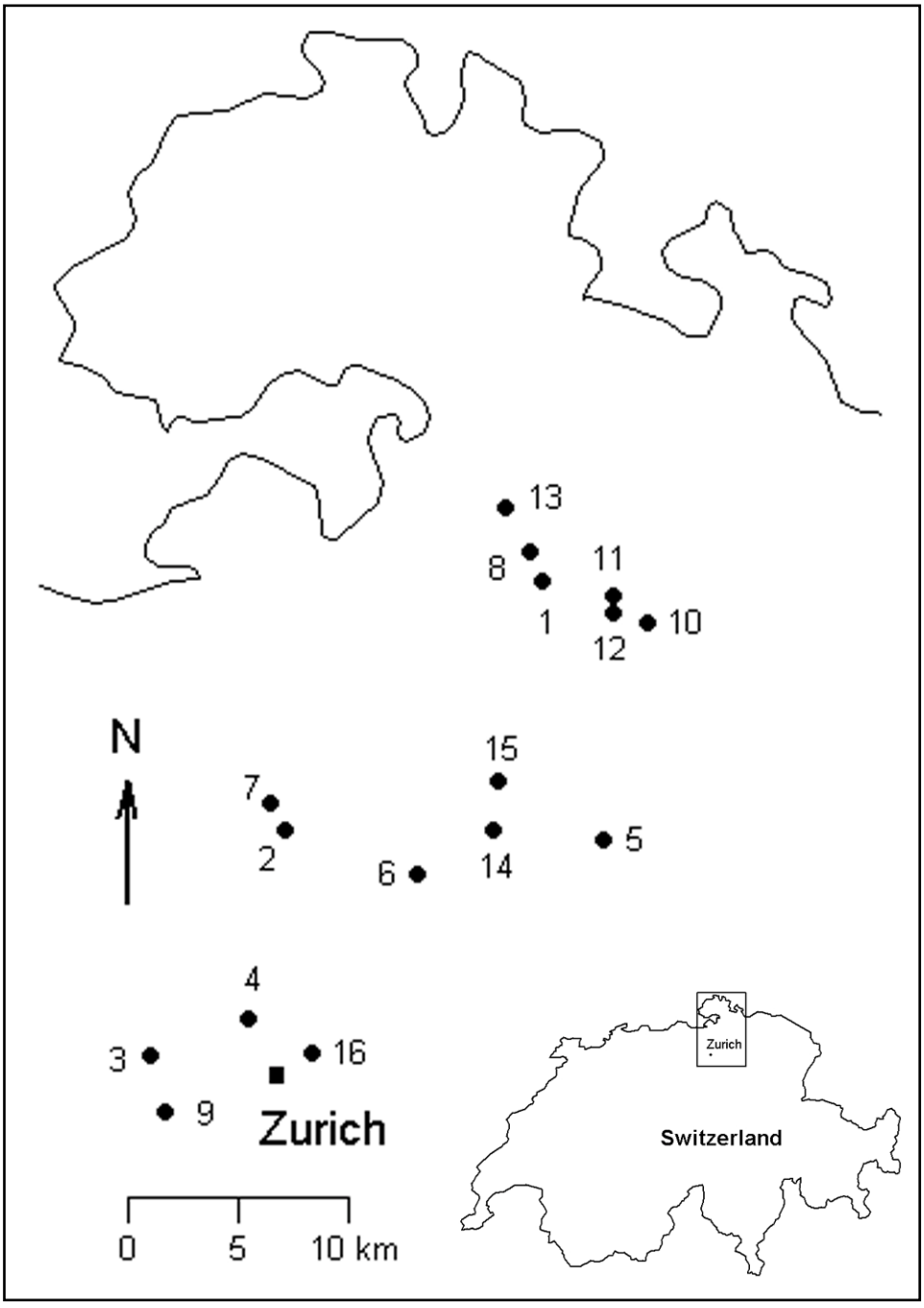
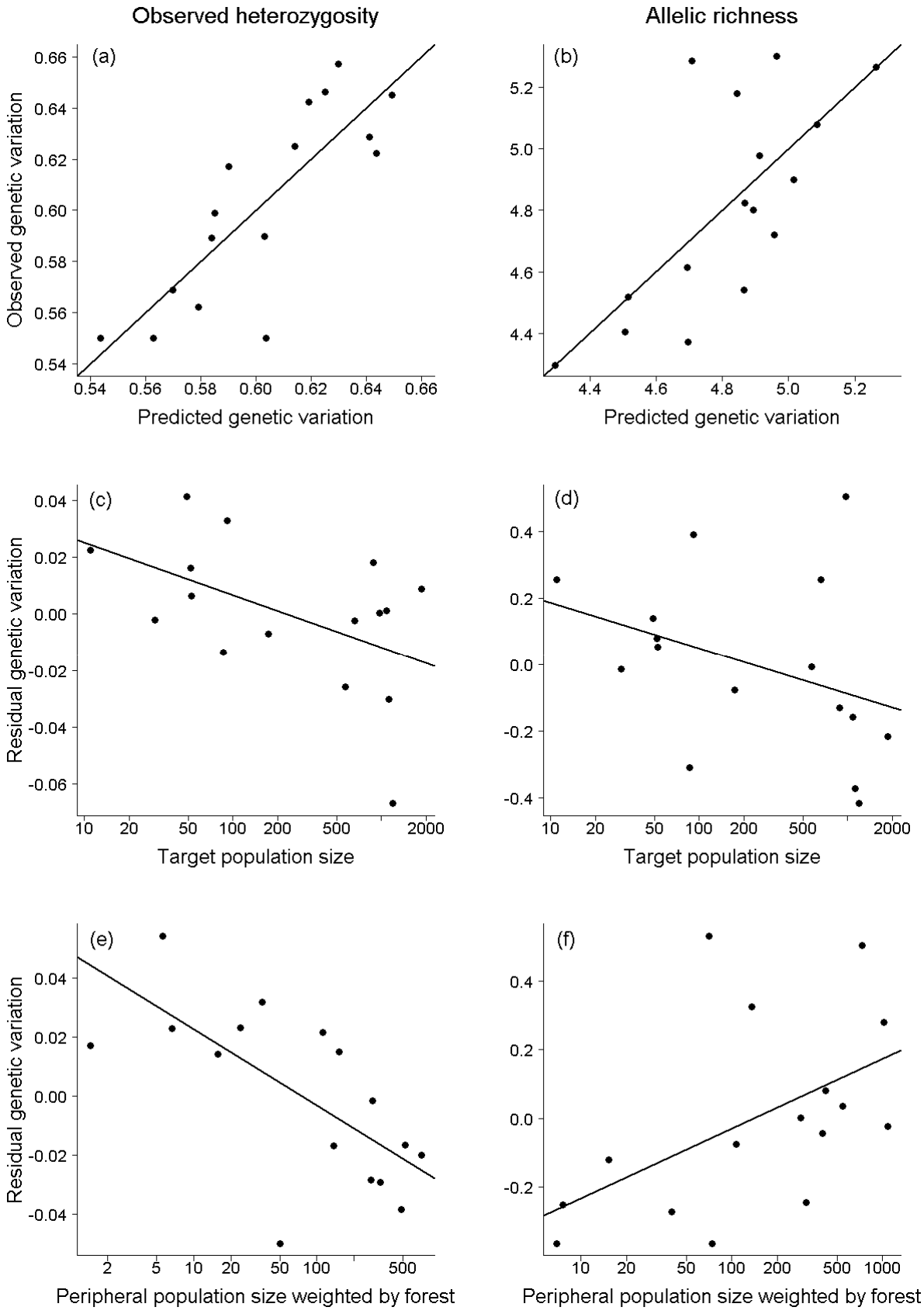


Fig. 2 – Observed and predicted values for genetic variation from the best models for observed heterozygosity H_o (left side of the figure) and allelic richness R_s (right side of the figure) (a and b), as well as the effect of the target population size (c and d) and peripheral population size weighted by forest (e and f). For H_o, predicted values were expressed as target population size and peripheral population sizes weighted by distance with sigmoid decrease and the impact of forest and roads (a). For R_s, predicted values were the target population size and peripheral population sizes weighed by distance as a decreasing linear function and forest and road impacts (b). In order to see the impact on the various components of the models, I fitted the models without the parameter of interest, kept the residuals, and plotted them on a log scale.


Observed heterozygosity
Allelic richness
(a)
(b)
(c)
(d)
(e)
(f)
Observed genetic variation
Predicted genetic variation
Residual genetic variation
Target population size
Peripheral population size weighted by forest

Appendices

Appendix A – Exact locations of the 16 *Rana temporaria* target populations, with population size and their cumulative peripheral population size.

Population (Code*)	Latitude (N)	Longitude (E)	Population size†	Peripheral size‡
Adlikon (1)	47° 34' 57"	8° 41' 58"	973	586 (7)
Allmend South (2)	47° 28' 49"	8° 32' 42"	30	2’208 (12)
Anni’s Pond (3)	47° 23' 17"	8° 27' 49"	883	11 (2)
Chäferberg (4)	47° 24' 13"	8° 31' 19"	575	284 (3)
Eschenberg (5)	47° 28’ 35’’	8° 44’ 13’’	174	776 (6)
Eigental (6)	47° 27’ 45’’	8° 37’ 28’’	52	1'601 (11)
Graben (7)	47° 29' 28"	8° 32' 10"	86	757 (6)
Hostbach (8)	47° 35' 41"	8° 41' 33"	49	2'402 (9)
Hueb West (9)	47° 21' 55’’	8° 28' 21"	1’872	181 (2)
Längeren (10)	47° 33' 56"	8° 45' 44"	92	2'619 (10)
Oberloo East (11)	47° 34’ 37’’	8° 44’ 32’’	1’078	3’478 (12)
Opfiker (12)	47° 34' 11"	8° 44' 31"	663	2'056 (11)
Räubrichseen (13)	47° 36’ 47’’	8° 40’ 37’’	53	2'259 (8)
Strubikon (14)	47° 28' 51"	8° 40' 10"	1'118	5'701 (7)
Weiertal (15)	47° 30’ 00’’	8° 40’ 21’’	11	6'817 (10)
Zürichberg (16)	47° 23’ 21’’	8° 33’ 41’’	1'193	170 (7)

* population codes correspond to the numbers on Fig.1

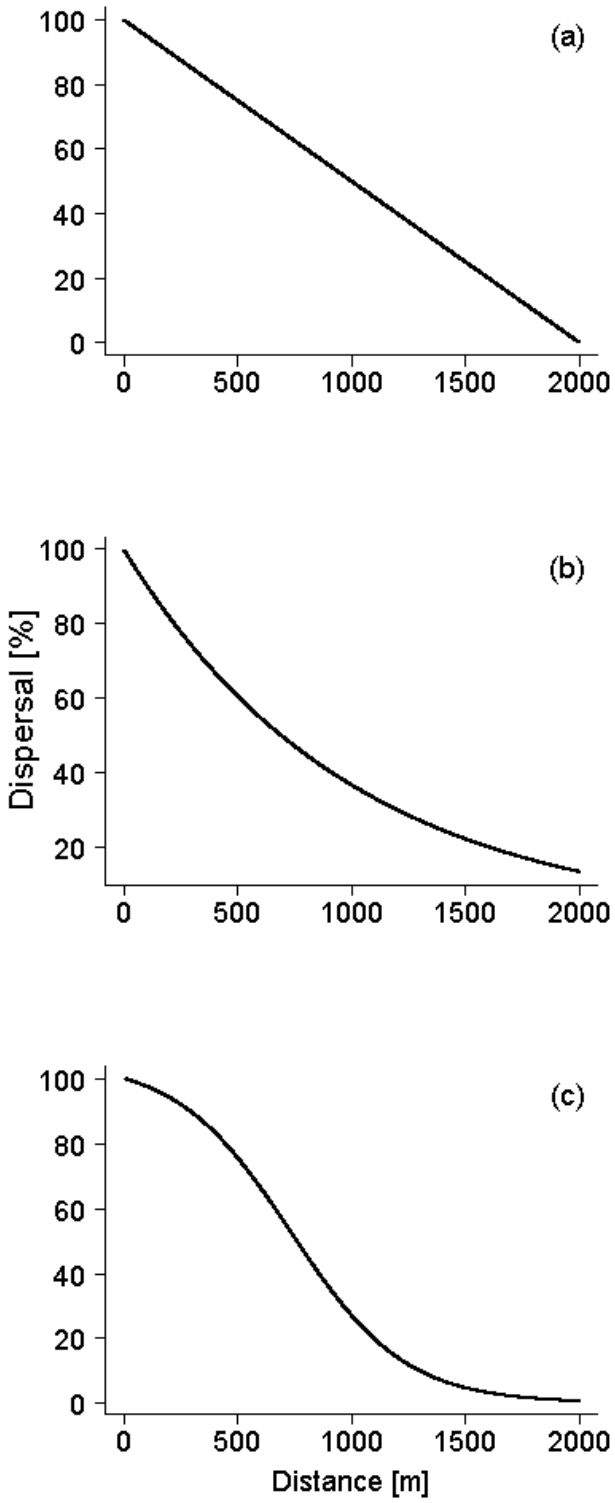
† population size of target populations: based on harmonic mean of egg clutch counts taken during 3-8 years between 1997 and 2009

‡ population size of peripheral populations, i.e. those within 2 km radius from the target population (in parentheses the number of total breeding sites): based on harmonic mean of egg clutch counts taken during 1-9 years between 1997 and 2009

Appendix B – List of the models for expressing genetic variation (observed heterozygosity H_o and allelic richness R_s) as function of target and peripheral population sizes. All population sizes were log-transformed after adding a value of one, to avoid problems with 0 values.

Model	Formula
Model 1	H_o / R_s ~ target population size
Model 2	H_o / R_s ~ target population size + Σ peripheral population sizes
Model 3	H_o / R_s ~ target population size + Σ relative population sizes
Model 4	H_o / R_s ~ target population size + Σ peripheral population sizes * (medium barriers)
Model 5	H_o / R_s ~ target population size + Σ peripheral population sizes * (large barriers)
Model 6	H_o / R_s ~ target population size + Σ peripheral population sizes * (medium + large barriers)
Model 7	H_o / R_s ~ target population size + Σ relative population sizes * (medium barriers)
Model 8	H_o / R_s ~ target population size + Σ relative population sizes * (large barriers)
Model 9	H_o / R_s ~ target population size + Σ relative population sizes * (medium + large barriers)
Model 10	H_o / R_s ~ target population size + Σ peripheral population sizes * distance (linear decrease)
Model 11	H_o / R_s ~ target population size + Σ peripheral population sizes * distance (exponential decrease)
Model 12	H_o / R_s ~ target population size + Σ peripheral population sizes * distance (sigmoid decrease)
Model 13	H_o / R_s ~ target population size + Σ relative population sizes * distance (linear decrease)
Model 14	H_o / R_s ~ target population size + Σ relative population sizes * distance (exponential decrease)
Model 15	H_o / R_s ~ target population size + Σ relative population sizes * distance (sigmoid decrease)
Model 16	H_o / R_s ~ target population size + Σ peripheral population sizes * distance (linear decrease) * land use
Model 17	H_o / R_s ~ target population size + Σ peripheral population sizes * distance (exponential decrease) * land use
Model 18	H_o / R_s ~ target population size + Σ peripheral population sizes * distance (sigmoid decrease) * land use
Model 19	H_o / R_s ~ target population size + Σ relative population sizes * distance (linear decrease) * land use
Model 20	H_o / R_s ~ target population size + Σ relative population sizes * distance (exponential decrease) * land use
Model 21	H_o / R_s ~ target population size + Σ relative population sizes * distance (sigmoid decrease) * land use

Appendix C – Expected dispersal in function of distance, expressed with three different functions: (a) linear decrease [-0.5*distance], (b) exponential decrease [$e^{-1*distance}$], and (c) sigmoid decrease (20 / [19+e4*distance]).



Appendix D – Genetic variation based on null allele adjusted frequencies at 5 microsatellite loci of the 16 Rana temporaria populations, with number of individuals scored (N), mean number of alleles per locus (A), allelic richness (R_S), mean observed and expected heterozygosity (H_o and H_e).

Population (Code*)	N	A (± SD)	R_S (± SD)	H_o (± SD)	H_e (± SD)
Adlikon (1)	40	7.6 ± 4.454	5.288 ± 2.512	0.625 ± 0.195	0.673 ± 0.203
Allmend South (2)	10	4.4 ± 2.871	4.295 ± 2.717	0.569 ± 0.236	0.564 ± 0.238
Anni's pond (3)	35	6.4 ± 4.758	4.405 ± 2.558	0.657 ± 0.251	0.617 ± 0.227
Chäferberg (4)	34	6.0 ± 3.633	4.519 ± 2.051	0.622 ± 0.143	0.648 ± 0.141
Eschenberg (5)	40	7.0 ± 4.290	4.902 ± 2.488	0.590 ± 0.214	0.639 ± 0.223
Eigental (6)	40	6.8 ± 4.956	4.753 ± 2.588	0.645 ± 0.167	0.627 ± 0.174
Graben (7)	40	7.4 ± 6.468	4.536 ± 3.053	0.550 ± 0.213	0.572 ± 0.223
Hostbach (8)	39	6.8 ± 4.622	5.079 ± 2.797	0.646 ± 0.154	0.661 ± 0.169
Hueb West (9)	40	6.6 ± 4.673	4.613 ± 2.538	0.617 ± 0.190	0.649 ± 0.177
Längeren (10	40	7.4 ± 4.454	5.181 ± 2.563	0.642 ± 0.211	0.682 ± 0.179
Oberloo East (11)	42	7.2 ± 4.792	4.825 ± 2.630	0.599 ± 0.215	0.629 ± 0.222
Opfiker (12)	37	7.8 ± 5.706	5.302 ± 3.018	0.589 ± 0.228	0.638 ± 0.232
Räubrichseen (13)	34	7.6 ± 5.083	4.977 ± 2.766	0.550 ± 0.248	0.604 ± 0.246
Strubikon (14)	37	7.6 ± 5.238	4.720 ± 2.572	0.562 ± 0.197	0.604 ± 0.188
Weiertal (15)	21	6.4 ± 3.411	5.267 ± 2.576	0.629 ± 0.129	0.692 ± 0.188
Zürichberg (16)	40	6.0 ± 3.847	4.373 ± 2.102	0.550 ± 0.177	0.595 ± 0.205

* population codes correspond to the numbers on Fig.1

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“It is the nature of all greatness not to be exact”.

Edmund Burke (1729-1797)

CURRICULUM VITAE

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